REMARKS

Rejection of the claims under 35 USC § 112:

Claims 7-11 and 24-28 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite. The action states that it is unclear if the reactive groups are still present of have been lost when they reacted. Applicants have amended the claim to obviate the rejection. Applicants have amended the claim to make the compound a product be process claim to remove the ambiguity.

The action also states that the term "reactive group" is indefinite. Applicants respectfully disagree. The term "reactive group" is readily recognizable to those skilled in the relevant art, organic chemistry. One skilled in the art would not consider any and all chemical compounds or groups to be reactive groups. Reactive groups are readily recognized in the art as groups which react with other groups to form covalent bonds under conditions which to do not break other bonds in the rest of the molecule. In support of this argument, Applicants provide pages 1053-1073 of Whitten et al, *General Chemistry with Qualitative Analysis.* This section provides a discussion of functional groups, which can be used interchangeably with the term reactive group. Further, Applicants have provided in the specification a listing a several exemplary reactive, or functional, groups (see for example page 5 line 18 to page 7 line 4).

Claims 24-28 have been rejected under 35 U.S.C. 112, first paragraph. The Action states that the phrase "that is activated by intramolecular attack from a free thiol such that it is cleaved more rapidly than oxidized glutathione" does not have support in the specification. The Action also states that there is no specific description nor guidance to what this phrase encompasses, nor how to generate this compound. Intramolecular attack and the structural requirements that facilitate intramolecular attack are a well-known in organic chemistry, as evidenced by Carey and Sundberg, *Advanced Organic Chemistry Second Edition*, pg. 147-150 and March, *Advanced Organic Chemistry Reactions, Mechanisms, and Structure*, pg 209-214. Intramolecular attack from thiols is discussed in Lin and Kim, *Biochemistry* 1989, Rabenstein and Yeo, *J. Org. Chem.* 1994, and Rabenstein and Waever *J. Org. Chem.* 1996. It is the Applicants' opinion that given the state of knowledge in the art, one skilled in the art would readily know how to construct a

compound containing a disulfide bond that is subject to intramolecular attach from a free thiol. Whether the resultant disulfide bond is cleaved faster than glutathione is then readily testable.

The Action further states that if a third thiol group existed in the composition, it is unclear how such a chemical would be made or exist in light of the potential for intramolecular attack. First, it is not a requirement that the composition initially contain a free thiol (third thiol group). The compound could easily contain a second disulfide bond. Cleavage of the second disulfide bond, either through spontaneous cleavage, enzymatic cleavage, or cleavage by another compound such as glutathione would generate a free thiol which would then be available for the intramolecular attack. Second, the compound could contain a protected thiol group. Removal of the protecting group, would then generate the free thiol. Third, the presence of a free thiol and a disulfide bond subject to intramolecular attack from the free thiol do not necessitate that the disulfide bond can not be formed or that it can not be sufficiently stable to allow use of a composition containing it. Because a reaction can occur does not require that the reaction be instantaneous. The rate at which the reaction occurs is determined by the kinetics of the reaction. The kinetics of a reaction can be altered by conditions such as temperature and solvent.

Rejection of the claims under 35 USC § 102:

Claims 7-11 and 19-28 have been rejected under 35 U.S.C. 102(b) as being anticipated by Pierce catalog as evidenced by Arpicco et al. Applicants have amended the claims to obviate the rejection. Support for electron withdrawing group is supported in the specification on page 2 line 17 to page 3 line 2, page 4 line 31 to page 5 line 9, page 14 lines 13-16, page 16 lines 25-28, and page 32 lines 8-12. Pierce does not teach a composition formed from reacting a compound containing a labile disulfide bond and at least one electron withdrawing group with molecules wherein the disulfide bond is located between at least two reactive groups and wherein the electron withdrawing group facilitates cleavage of the disulfide bond.

The Examiner's rejections are now believed to be overcome by this response to the Office Action. In view of Applicants' amendment and arguments, it is submitted that claims 7-11 and 19-26 should be allowable.

Respectfully submitted,

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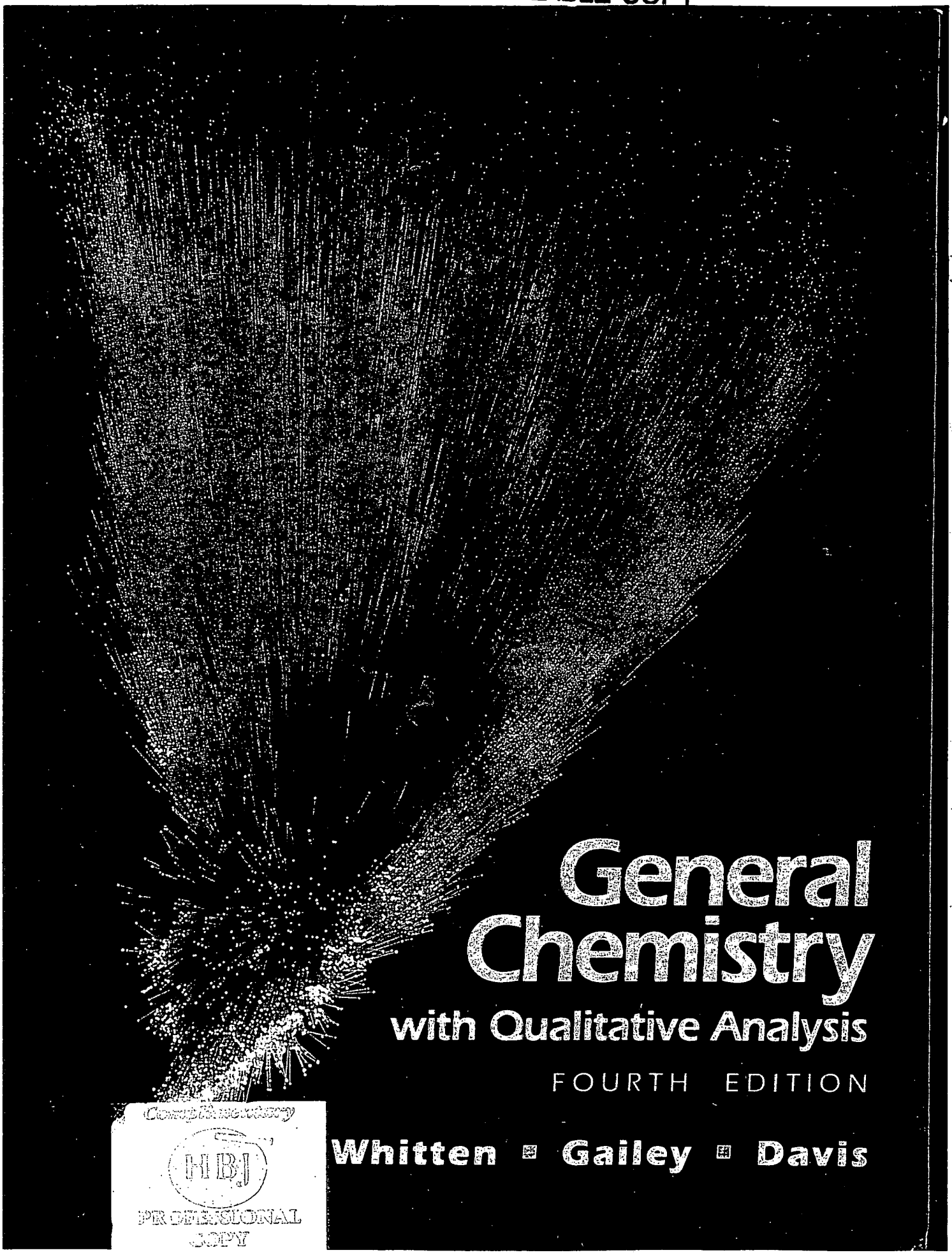
I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as express mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on this date: 5/27/05.

Kirk Ekena



General Chemistry

with Qualitative Analysis

FOURTH EDITION

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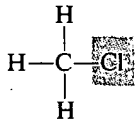
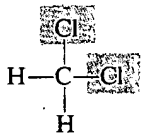
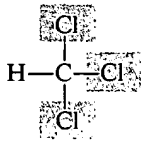
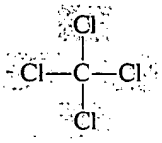
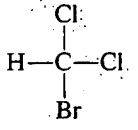
Functional Groups

The study of organic chemistry is greatly simplified by considering hydrocarbons as parent compounds and describing other compounds as derived from them. In general, an organic molecule consists of a skeleton of carbon atoms with special groups of atoms within or attached to that skeleton. These special groups of atoms are often called **functional groups** because they represent the most common sites of chemical reactivity (function). The only functional groups that are possible in hydrocarbons are double and triple (i.e., pi) bonds. Atoms other than C and H are called **heteroatoms**, the most common being O, N, S, P, and the halogens. Most functional groups contain one or more heteroatoms.

As you study the following sections, you may wish to refer to the summary in Section 31-15.

In the next several sections, we shall introduce some common functional groups that contain heteroatoms and learn a little about the resulting classes of compounds. We shall continue to represent hydrocarbon groups with the symbol R—. We commonly use that symbol to represent either an aliphatic (e.g., alkyl) or an aromatic (e.g., an aryl such as phenyl) group. When we specifically mean an aryl group, we shall use the symbol Ar—.

Table 31-7 Some Organic Halides

Formula	Structural Formula	Normal bp (°C)	IUPAC Name	Common Name
CH_3Cl		23.8	chloromethane	methyl chloride
CH_2Cl_2		40.2	dichloromethane	methylene chloride
$CHCl_3$		61	trichloromethane	chloroform
CCl_4		76.8	tetrachloromethane	carbon tetrachloride
$CHCl_2Br$		90	bromodichloromethane	—

31-8 Organic Halides

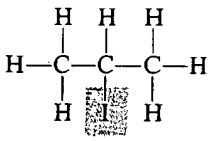
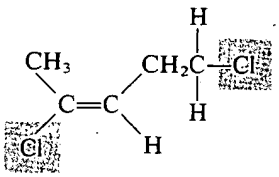
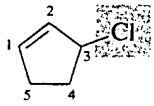
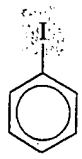
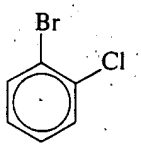
Almost any hydrogen atom in a hydrocarbon can be replaced by a halogen atom to give a stable compound. Table 31-7 shows some organic halides and their names.

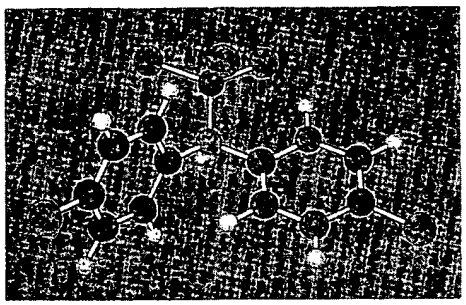
In the IUPAC naming system, the organic halides are named as *halo-* derivatives of the parent hydrocarbons. The prefix *halo-* can be *fluoro-*, *chloro-*, *bromo-*, or *iodo-*. Simple alkyl chlorides are sometimes given common names as alkyl derivatives of the hydrogen halides. For instance, the IUPAC name for CH_3CH_2—Cl is chloroethane; it is commonly called ethyl chloride by analogy to H—Cl, hydrogen chloride.

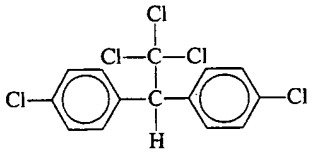
A carbon atom can be bonded to as many as four halogen atoms, so an enormous number of organic halides can exist. Completely fluorinated compounds are known as **fluorocarbons** or sometimes *perfluorocarbons*. The fluorocarbons are even less reactive than hydrocarbons. Saturated compounds in which all H atoms have been replaced by some combination of Cl and F atoms are called *chlorofluorocarbons* or sometimes **freons**. These compounds have been widely used as refrigerants and as propellants in

Freon is a Du Pont trademark for certain chlorofluorocarbons; other companies' related products are known by other names. Typical freons are trichlorofluoromethane, $CFCl_3$ (called Freon-11), and dichlorodifluoromethane, CF_2Cl_2 (called Freon-12).

Table 31-7 (continued)

Formula	Structural Formula	Normal bp (°C)	IUPAC Name	Common Name
$(CH_3)_2CHI$		89.5	2-iodopropane	isopropyl iodide
$CH_3ClC{=}CHCH_2CH_2Cl$		40	2,5-dichloro-*cis*-2-pentene; *trans* isomer is also possible	—
C_5H_7Cl		25	3-chlorocyclopentene is shown; other isomers are also possible	—
C_6H_5I		118	iodobenzene	phenyl iodide
C_6H_4ClBr		204	1-bromo-2-chlorobenzene is shown; other isomers are also possible	*o*-bromochlorobenzene





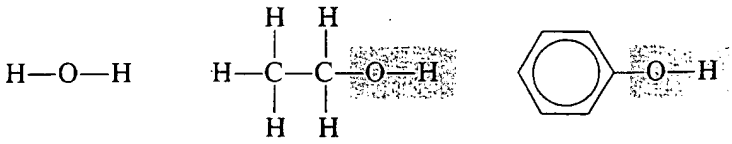
DDT, an organic halide whose full name is 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane. It was introduced as an insecticide in the 1940s and widely used until the early 1970s. This compound was almost entirely responsible for the virtual eradication of malaria, once the world's most widespread disease, by killing the *Anopheles* mosquito that carried the disease. Unfortunately, this toxic substance stays in the environment for a long time. As a result, it is carried up the food chain and concentrates in the bodies of higher animals. It is especially detrimental in the life cycles of birds. Only about 2% of the DDT in the environment is degraded each year. Even though its use has been banned for many years, it will still take many years before the last traces disappear from the soil.

aerosol cans. However, the release of chlorofluorocarbons into the atmosphere has been shown to be quite damaging to the earth's ozone layer. Since January 1978 the use of chlorofluorocarbons in aerosol cans in the United States has been banned, and efforts to develop both controls for existing chlorofluorocarbons and suitable replacements continue.

31-9 Alcohols and Phenols

Alcohols and phenols contain the hydroxyl group (—O—H) as their functional group. **Alcohols** may be considered to be derived from saturated or unsaturated hydrocarbons by the replacement of at least one H atom by a hydroxyl group. The properties of alcohols result from a hydroxyl group attached to an *aliphatic* carbon atom, —C—O—H. Ethanol (ethyl alcohol) is the most common example (Figure 31-14).

When a hydrogen atom on an aromatic ring is replaced by a hydroxyl group (Figure 31-15), the resulting compound is known as a **phenol**. Such compounds behave more like acids than alcohols. Alternatively, we may view alcohols and phenols as derivatives of water in which one H atom has been replaced by an organic group:



water ethanol phenol

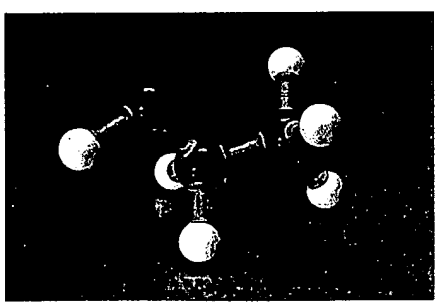
Indeed, this is a better view. The structure of water was discussed in Section 8-9. The hydroxyl group in an alcohol or a phenol is covalently bonded to a carbon atom, but the O—H bond is quite polar. The oxygen atom has two unshared electron pairs, and the C—O—H bond angle is nearly 104.5°.

The presence of a bonded alkyl or aryl group changes the properties of the —OH group. *Alcohols* are so very weakly acidic that they are thought of as neutral compounds. *Phenols* are weakly acidic.

Many properties of alcohols depend on whether the hydroxyl group is attached to a carbon that is bonded to *one*, *two*, or *three* other carbon atoms.

The simplest phenol is called phenol. The most common member of a class of compounds is often called by the class name. Salt, sugar, alcohol, and phenol are examples.

Primary alcohols contain one R group, **secondary alcohols** contain two R groups, and **tertiary alcohols** contain three R groups bonded to the carbon atom to which the —OH group is attached.



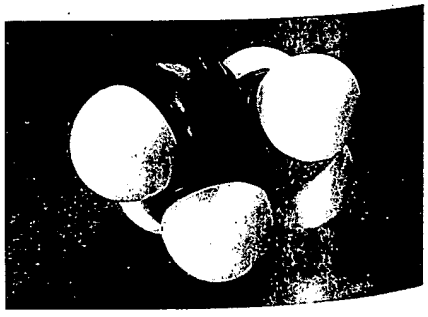
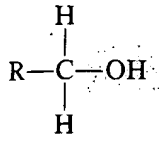
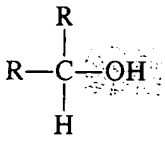


Figure 31-14
Models of ethanol (also called ethyl alcohol or grain alcohol), CH_3CH_2OH.

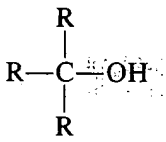
esenting alkyl groups as R, we can illustrate the three classes of alco- The R groups may be the same or different.



primary (1°) alcohol



a secondary (2°) alcohol

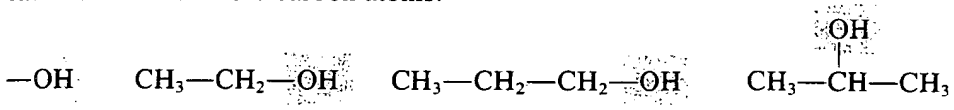


a tertiary (3°) alcohol

In writing organic structures, we often use primes which we wish to specify that the alkyl groups might be different, e.g., R, R', R".

ing Alcohols and Phenols

systematic name of an alcohol consists of the characteristic stem plus *l* ending. A numeric prefix indicates the position of the —OH group chain of three or more carbon atoms.



hano
l alcohol
alcohol)

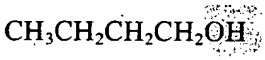
ethanol
ethyl alcohol
(grain alcohol)

1-propanol
n-propyl alcohol
(a primary alcohol)

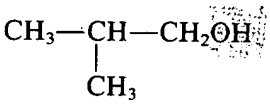
2-propanol
isopropyl alcohol
(a secondary alcohol)

are four structural isomers of the saturated acyclic four-carbon al- s with one —OH per molecule.

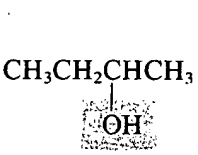
Acyclic compounds contain no rings.

1°: 

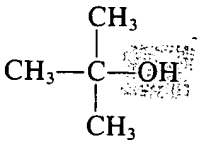
1-butanol
normal butyl alcohol

1°: 

2-methyl-1-propanol
isobutyl alcohol

2°: 

2-butanol
secondary butyl alcohol

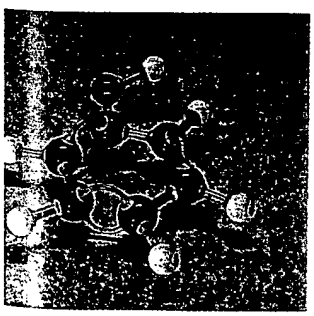
3°: 

2-methyl-2-propanol
tertiary butyl alcohol

are eight structural isomers of the analogous five-carbon alcohols. are often called "amyl" or "pentyl" alcohols. Two examples are

1-pentanol
n-pentyl alcohol
n-amyl alcohol

3-methyl-1-butanol
isopentyl alcohol
isoamyl alcohol



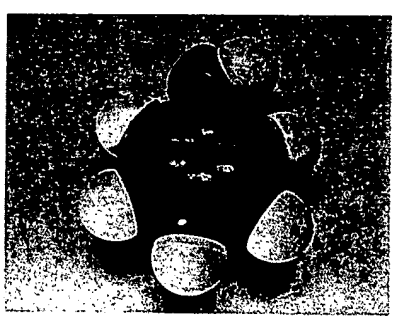
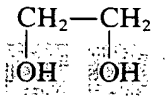


Figure 31-15
Models of phenol, C_6H_5OH.

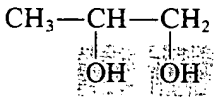
Polyhydric alcohols are used in permanent antifreeze and in cosmetics.

The *o*-, *m*-, and *p*- notation was introduced in Section 31-6.

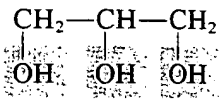
The **polyhydric alcohols** contain more than one —OH group per molecule. Those containing two OH groups per molecule are called **glycols**. Important examples of polyhydric alcohols include



1,2-ethanediol
ethylene glycol
(the major ingredient in permanent antifreeze)

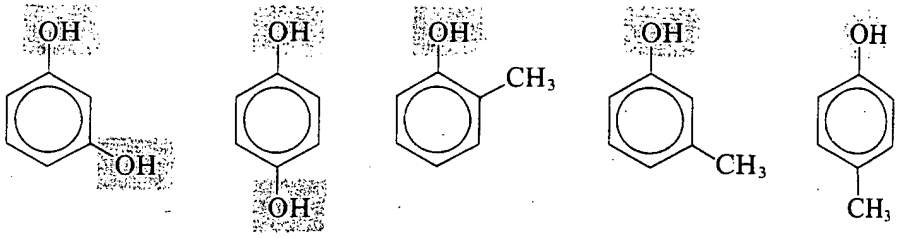


1,2-propanediol
propylene glycol



1,2,3-propanetriol
glycerine or glycerol
(a moisturizer in cosmetics)

Phenols are usually referred to by their common names. Examples are

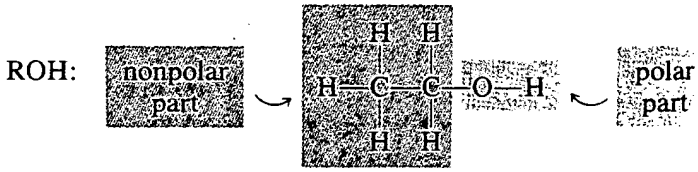


resorcinol hydroquinone *o*-cresol *m*-cresol *p*-cresol

As you might guess, cresols occur in "creosote," a wood preservative.

Physical Properties of Alcohols and Phenols

The hydroxyl group, —OH, is quite polar, whereas alkyl groups, R, are nonpolar. The properties of alcohols depend on two factors: (1) the number of hydroxyl groups per molecule and (2) the size of the nonpolar portion of the molecule.



The low-molecular-weight monohydric alcohols are miscible with water in all proportions. Beginning with the four butyl alcohols, solubility in water decreases rapidly with increasing molecular weight. This is because the nonpolar parts of such molecules are much larger than the polar parts. Many

Table 31-8
Physical Properties of Normal Primary Alcohols

Name	Formula	Normal bp (°C)	Solubility in H_2O (g/100 g at 20°C)
methanol	CH_3OH	65	Completely miscible
ethanol	CH_3CH_2OH	78.5	Completely miscible
1-propanol	$CH_3CH_2CH_2OH$	97	Completely miscible
1-butanol	$CH_3CH_2CH_2CH_2OH$	117.7	7.9
1-pentanol	$CH_3CH_2CH_2CH_2CH_2OH$	137.9	2.7
1-hexanol	$CH_3CH_2CH_2CH_2CH_2CH_2OH$	155.8	0.59

lyhydric alcohols are very soluble in water because they contain two or ıre polar —OH groups per molecule.

Table 31-8 shows that the boiling points of normal primary alcohols in- :ase, and their solubilities in water decrease, with increasing molecular ight. The boiling points of the alcohols are much higher than those of the responding alkanes (Table 31-2) because of the hydrogen bonding of the lroxyl groups.

Most phenols are solids at 25°C. Phenols are only slightly soluble in water ess they contain other functional groups that interact with water.

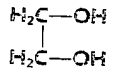
ne Uses of Alcohols and Phenols

ny alcohols and phenols have considerable commercial importance. thanol, CH_3OH, was formerly produced by the destructive distillation of od and is sometimes called wood alcohol. It is now produced in large ntities from carbon monoxide and hydrogen. It is extensively used as a vent for varnishes and shellacs, as the starting material in the manufacture ormaldehyde (Section 31-14), as a temporary antifreeze (bp = 65°C), and ı fuel additive. It is very toxic and causes permanent blindness when :n internally.

:thanol, CH_3CH_2OH, also known as ethyl alcohol or grain alcohol, was prepared by fermentation *a long time ago*. The most ancient written ature refers to beverages that were obviously alcoholic! The syrupy due from the purification of cane sugar (sucrose) is blackstrap molasses. 'ermentation is one important source of ethanol. The starches in grains, ıtoes, and similar foodstuffs can be converted into sugar by malt; this is ıwed by fermentation to produce ethanol, which is the most important ıstrial alcohol. Like the other shorter-chain alcohols, ethanol participates ydrogen bonding and is completely miscible with water.

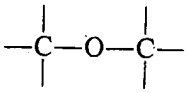
Fermentation is an enzymatic process carried out by certain kinds of bacteria.

lany simple alcohols are important raw materials in the industrial syn- is of polymers, fibers, explosives, plastics, and pharmaceutical products. ıols are found in plant products such as flower pigments, tanning agents, wood. They are widely used in the preparation of plastics and dyes. te aqueous solutions of phenols are used as antiseptics and disinfectants. hydric alcohols are useful for their relatively high boiling points. For ınce, glycerine is used as a wetting agent in cosmetic preparations. 'lene glycol (bp = 197°C), which is completely miscible with water, is in commercial permanent antifreeze.


ethylene glycol

10 Ethers

n the word "ether" is mentioned, most people think of the well-known thetic, diethyl ether. There are many ethers. Their uses range from cial flavorings to refrigerants and solvents. An **ether** is a compound in h an O atom is bonded to two organic groups:



ıols are considered derivatives of water in which one H atom has been ced by an organic group. Ethers may be considered derivatives of water ıich both H atoms have been replaced by organic groups.

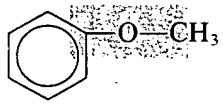
H—O—H R—O—H R—O—R′
water alcohol ether

However, the similarity is only structural because ethers are not very polar and are chemically rather unreactive. (We shall not discuss their reactions here.) In fact, their physical properties are similar to those of the corresponding alkanes; e.g., CH_3OCH_3 is like $CH_3CH_2CH_3$.

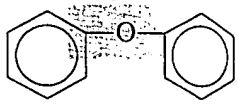
Three kinds of ethers are known: (1) aliphatic, (2) aromatic, and (3) mixed. Common names are used for ethers in most cases.

methoxymethane
dimethyl ether
(an aliphatic ether)

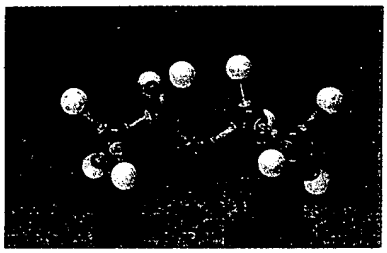
methoxyethane
methyl ethyl ether
(an aliphatic ether)

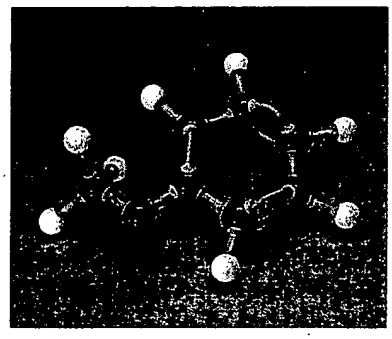


methoxybenzene
methyl phenyl ether
anisole
(a mixed ether)



phenoxybenzene
diphenyl ether
(an aromatic ether)





Models of diethyl ether (top) and methyl phenyl ether (bottom).

Diethyl ether is a very-low-boiling liquid (bp = 35°C). Dimethyl ether is a gas that is used as a refrigerant. The aliphatic ethers of higher molecular weights are liquids, and the aromatic ethers are liquids and solids.

Even ethers of low molecular weight are only slightly soluble in water. Diethyl ether is an excellent solvent for organic compounds. It is widely used to extract organic compounds from plants and other natural sources.

Ethers burn readily, and care must be exercised to avoid fires when ethers are used. At room temperature diethyl ether is oxidized by oxygen in the air to a nonvolatile, explosive peroxide. Thus, ethereal solutions should never be evaporated to dryness, because of the danger of peroxide explosions, unless proper precautionary steps have been taken to destroy all peroxides in advance.

31-11 Amines

The **amines** are derivatives of ammonia in which one or more hydrogen atoms have been replaced by alkyl or aryl groups. Many low-molecular-weight amines are gases or low-boiling liquids (Table 31-9). Amines are basic compounds (Table 18-6; Section 32-7). Their basicity differs, depending on the nature of the organic substituents. The aliphatic amines of low molecular weight are soluble in water. Aliphatic diamines of fairly high molecular

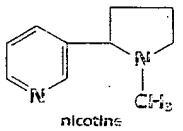
Ammonia acts as a Lewis base because there is one unshared pair of electrons on the N atom (Section 10-10).

Table 31-9
Boiling Points of Some Amines

Name	Formula	Boiling Point (°C)
ammonia	NH_3	−33.4
methylamine	CH_3NH_2	−6.5
dimethylamine	$(CH_3)_2NH$	7.4
trimethylamine	$(CH_3)_3N$	3.5
ethylamine	$CH_3CH_2NH_2$	16.6
aniline	$C_6H_5NH_2$	184
ethylenediamine	$H_2NCH_2CH_2NH_2$	116.5
pyridine	C_5H_5N	115.3

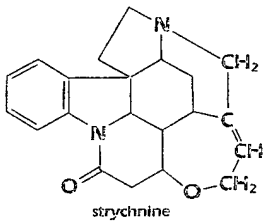
weight are soluble in water because each molecule contains two highly polar $-NH_2$ groups that form hydrogen bonds with water.

The odors of amines are quite unpleasant; many of the malodorous compounds that are released as fish decay are simple amines. Amines of high molecular weight are nonvolatile, so they have little odor. One of the materials used to manufacture nylon, hexamethylenediamine, is an aliphatic amine. Many aromatic amines are used to prepare organic dyes that are widely used in industrial societies. Amines are also used to produce many medicinal products, including local anesthetics and sulfa drugs.



nicotine

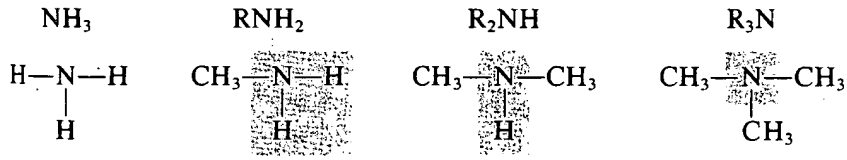
Amines are widely distributed in nature in the form of amino acids and proteins, which are found in all higher animal forms, and in alkaloids, which are found in most plants. Some of these substances are fundamental building blocks of animal tissue, and minute amounts of others have dramatic physiological effects, both harmful and beneficial. Countless other biologically important substances, including many vitamins, antibiotics, and drugs, contain amino groups, $—NR_2$ (where R can represent an H, alkyl, or aryl group).



strychnine

Structure and Naming of Amines

There are three classes of amines, depending on whether one, two, or three hydrogen atoms have been replaced by organic groups. They are called primary, secondary, and tertiary amines, respectively.



ammonia | methylamine (a primary amine) | dimethylamine (a secondary amine) | trimethylamine (a tertiary amine)

Models of these four molecules are shown in Figure 31-16.

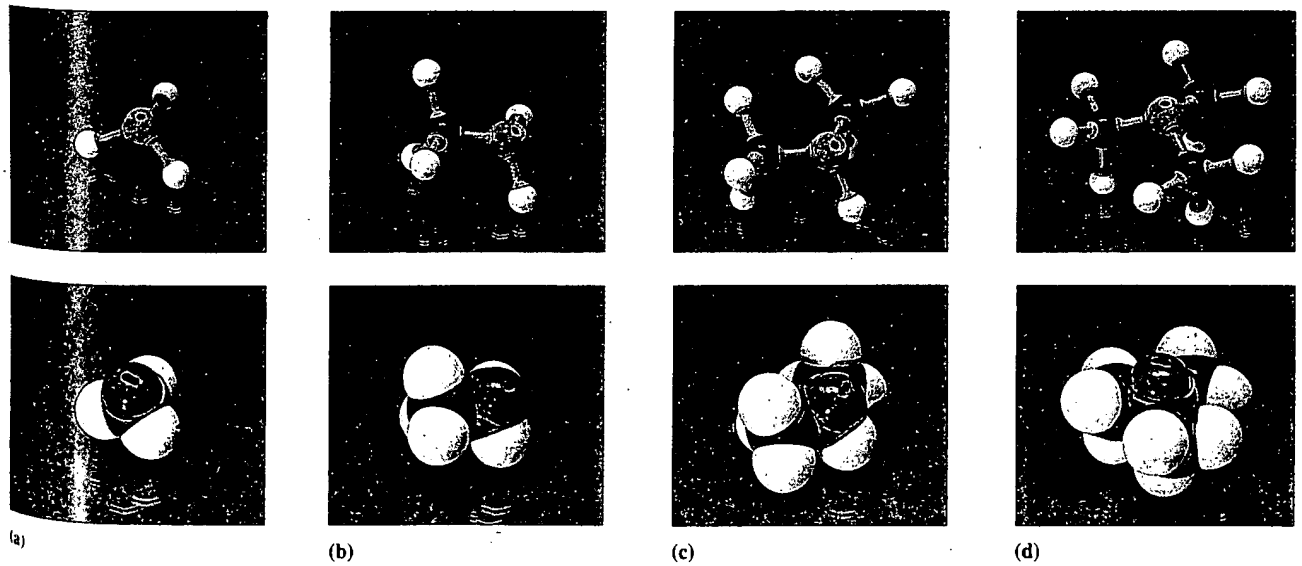
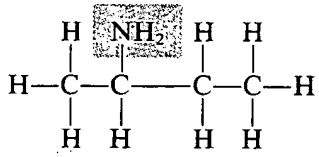


Figure 31-16
Models of (a) ammonia, (b) methylamine, (c) dimethylamine, and (d) trimethylamine.

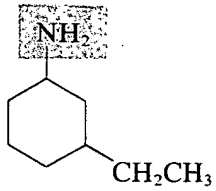
groups are trying to find the conditions necessary for encapsulating atoms with C_{60} or with other fullerenes that have larger cavities. The unusual shape of the C_{60} molecule is of special interest in situations in which molecular shapes determine chemical activity, as in biological molecules, pharmaceutical drugs, and polymers. To investigate these kinds of applications, other functional groups or reactive organic systems have already been attached to C_{60}. Long chains of the form $(C_{60})—R—(C_{60})—R—\ldots$ have also been constructed. This peculiar new molecule, C_{60}, and other members of its family are rapidly emerging from the realm of molecular beams into the mainstream of practical chemistry.

Professor Michael A. Duncan
The University of Georgia

The systematic names of amines are based on consideration of the compounds as derivatives of ammonia. Amines of more complex structure are sometimes named as derivatives of the parent hydrocarbon, with the term *amino-* used as a prefix to describe $—NH_2$.

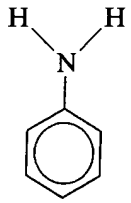


2-aminobutane or *sec*-butylamine

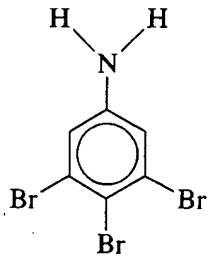


1-amino-3-ethylcyclohexane

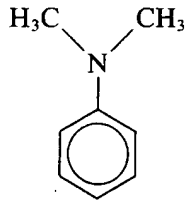
Aniline is the simplest aromatic amine. Many aromatic amines are named as derivatives of aniline.



aniline (primary)

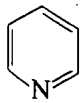


3,4,5-tribromoaniline

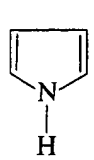


N,N-dimethylaniline

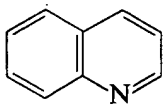
Heterocyclic amines contain nitrogen as a part of the ring, bound to two carbon atoms. Many of these amines are found in coal tar and a variety of natural products. Some aromatic and heterocyclic amines are always called by their common names.



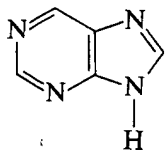
pyridine (tertiary)



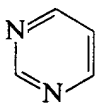
pyrrole (secondary)



quinoline (tertiary)



purine

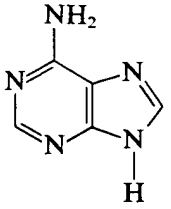


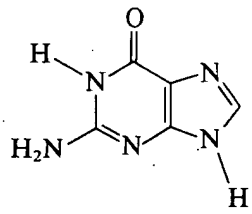
pyrimidine

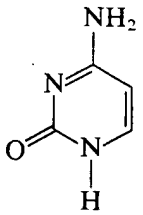
Genes, the units of chromosomes that carry hereditary characteristics, are essentially long stretches of double helical deoxyribonucleic acid, or DNA. DNA is composed of four fundamental *nucleotide bases*: adenine, guanine, cytosine, and thymine. The first two are modified purines, and the latter two are modified pyrimidines. The sequence of these building blocks

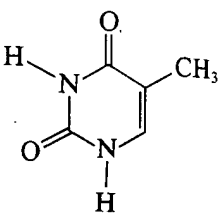
A space-filling model of a portion of the DNA double helical structure.

in DNA acts as a code for the order of amino acids in the proteins of an organism. The DNA in each cell of an organism contains the instructions for making the complete organism.

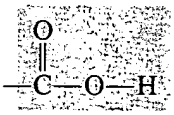

adenine


guanine


cytosine


thymine

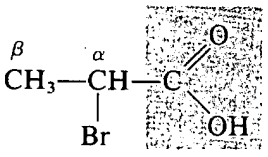
31-12 Carboxylic Acids

Compounds that contain the **carboxyl group**,  , are acidic. They are called **carboxylic acids**. Their general formula is R—COOH. Most are *weak acids* compared with strong inorganic acids such as HCl. However, they are much stronger acids than most phenols. Carboxylic acids are named systematically by dropping the terminal *-e* from the name of the parent hydrocarbon and adding *-oic acid* (Table 31-10). However, many older names survive, and organic acids are usually called by common names. In aromatic acids the carboxyl group is attached to an aromatic ring (Figure 31-17).

Aliphatic carboxylic acids are sometimes referred to as *fatty acids* because many have been obtained from animal fats.

Organic acids occur widely in natural products, and many have been known since ancient times. Their common (trivial) names are often derived from a Greek or Latin word that indicates the original source (Table 31-10).

The names of derivatives of carboxylic acids are often derived from the trivial names of the acids. Positions of substituents are sometimes indicated by lowercase Greek letters, beginning with the carbon *adjacent* to the carboxyl carbon, rather than by numbering the carbon chain.


2-bromopropanoic acid
(α-bromopropionic acid)

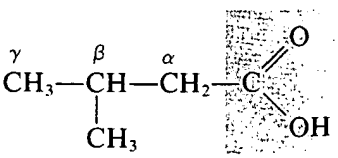
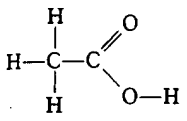

3-methylbutanoic acid
(β-methylbutyric acid)

Table 31-10
Some Aliphatic Carboxylic Acids

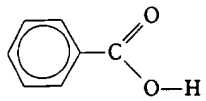
Formula	Common Name	IUPAC Name
HCOOH	formic acid	methanoic acid
CH_3COOH	acetic acid	ethanoic acid
CH_3CH_2COOH	propionic acid	propanoic acid
$CH_3CH_2CH_2COOH$	*n*-butyric acid	butanoic acid
$CH_3CH_2CH_2CH_2CH_2COOH$	*n*-caproic acid	hexanoic acid
$CH_3(CH_2)_{10}COOH$	lauric acid	dodecanoic acid
$CH_3(CH_2)_{14}COOH$	palmitic acid	hexadecanoic acid
$CH_3(CH_2)_{16}COOH$	stearic acid	octadecanoic acid

Formic acid was obtained by distillation of ants (L. *formica*, ant); acetic acid occurs in vinegar (L. *acetum*, vinegar); butyric acid in rancid butter (L. *butyrum*, butter); stearic acid in animal fats (Gr. *stear*, beef suet). *n*-Caproic acid is one of the so-called "goat acids." Its odor is responsible for the name.

a) acetic acid
(an aliphatic acid)



b) benzoic acid
(an aromatic acid)



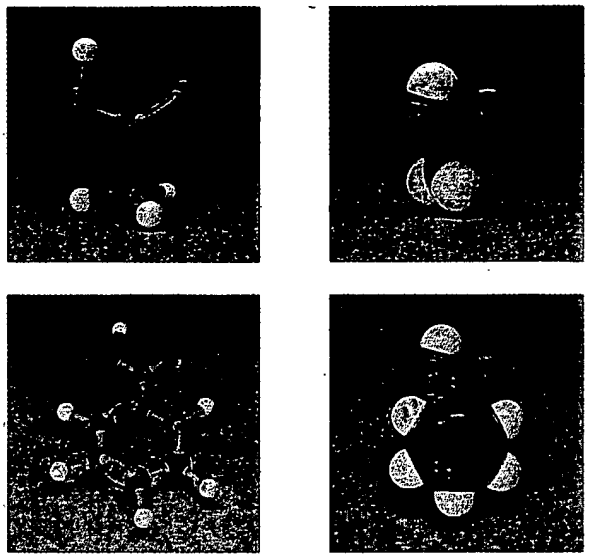
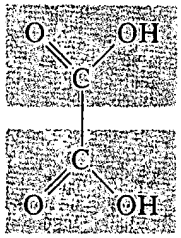
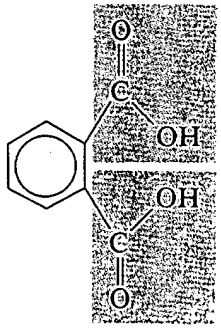


Figure 31-17
(a) Models of acetic acid. (b) Models of benzoic acid.

Some carboxylic acid molecules contain more than one —COOH group Table 31-11). These acids are nearly always called by their common names.)xalic acid is an aliphatic **dicarboxylic acid**, and phthalic acid is a typical romatic dicarboxylic acid.



oxalic acid
(an aliphatic acid)

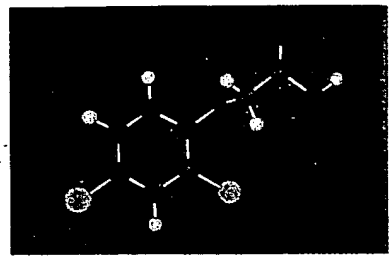


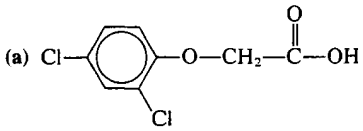
phthalic acid
(an aromatic acid)

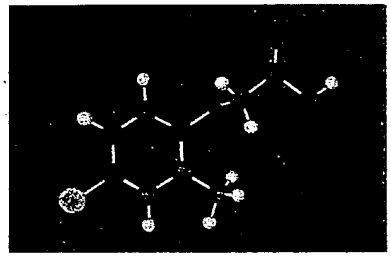
Aromatic acids are called by their common names or named as derivatives f benzoic acid, which is considered the "parent" aromatic acid.

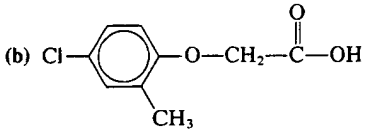
Table 31-11
Some Aliphatic Dicarboxylic Acids

Formula	Name
HOOC—COOH	oxalic acid
HOOC—CH_2—COOH	malonic acid
HOOC—CH_2CH_2—COOH	succinic acid
HOOC—$CH_2CH_2CH_2$—COOH	glutaric acid
HOOC—$CH_2CH_2CH_2CH_2$—COOH	adipic acid



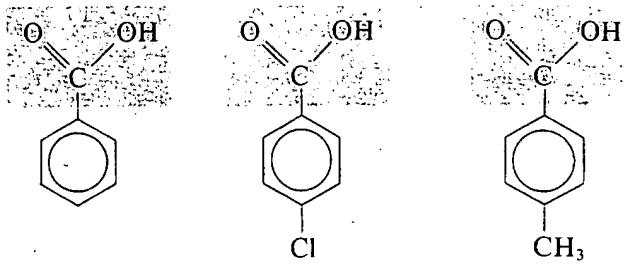
(a) 



(b) 

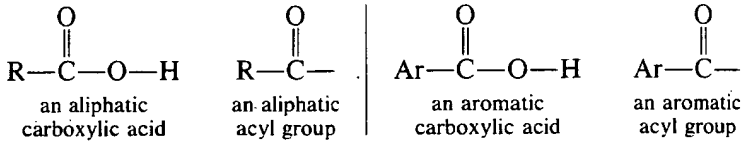
These two derivatives of phenoxyacetic acids act as herbicides (weed killers) by overstimulating the plant's growth system.

Crystals of glycine viewed under polarized light. Glycine, the simplest amino acid, has the structure shown in the text, with R = H.



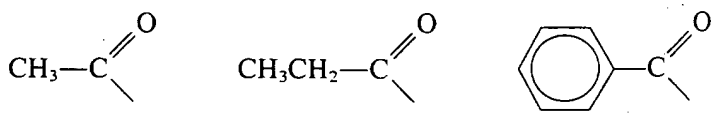
benzoic acid | *p*-chlorobenzoic acid | *p*-toluic acid

Many reactions of carboxylic acids involve displacement of the —OH group by another atom or group of atoms. We find it useful to name the non-OH portions of acid molecules because they occur in many compounds. Such compounds are thought of as derivatives of carboxylic acids.



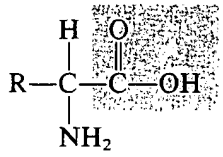
an aliphatic carboxylic acid | an aliphatic acyl group | an aromatic carboxylic acid | an aromatic acyl group

Acyl groups are named as derivatives of the parent acid by dropping *-ic acid* and adding *-yl* to the characteristic stem. Some examples are



acetyl group | propionyl group | benzoyl group

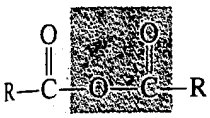
Although many carboxylic acids occur in the free state in nature, many occur as amides or esters (Sections 31-13). Amino acids are substituted carboxylic acids with the general structure



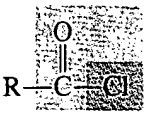
where R can be either an alkyl or an aryl group. Amino acids are the components of proteins, which make up the muscle and tissue of animals. Many other acids are important in the metabolism and synthesis of fats by enzyme systems. Acetic acid (the acid in vinegar) is the end product in the fermentation of most agricultural products. It is the fundamental unit used by living organisms in the biosynthesis of such widely diverse classes of natural products as long-chain fatty acids, natural rubber, and steroid hormones. It is also a powerful solvent, and an important reagent in the preparation of pharmaceuticals, plastics, artificial fibers, and coatings. Phthalic acid and adipic acid are used in the production of synthetic polymers that are used as fibers (e.g., Dacron and nylon, Section 32-11).

31-13 Some Derivatives of Carboxylic Acids

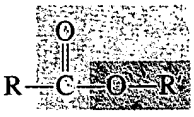
Four important classes of acid derivatives are formed by the replacement of the hydroxyl group by another atom or group of atoms. Each of these derivatives contains an acyl group.



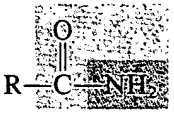
an acid anhydride



an acyl chloride (an acid chloride)



an ester



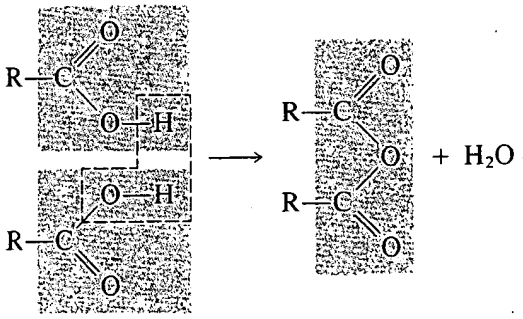
an amide

Aromatic compounds of these types (with R = aryl groups) are encountered frequently.

Acid Anhydrides

An acid anhydride can be thought of as the result of removing one molecule of water from two carboxylic acid groups.

The structural relationship between monocarboxylic acids and their anhydrides is

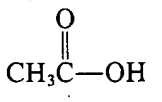


two molecules of a carboxylic acid

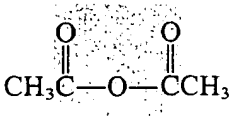
one molecule of acid anhydride

Acid anhydrides are usually prepared by indirect methods. This illustration shows the structural relationship that is important for our purposes.

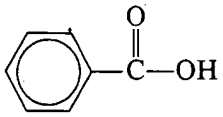
The anhydrides are named by replacing the word "acid" in the name of the parent acid with the word "anhydride." Examples of acids and their anhydrides are



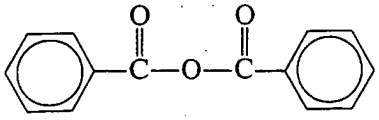
acetic acid



acetic anhydride



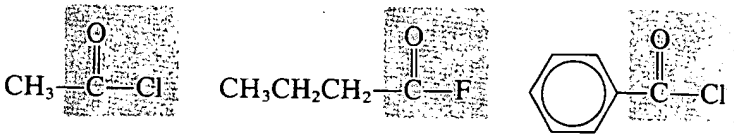
benzoic acid



benzoic anhydride

Acyl Halides (Acid Halides)

The **acyl halides**, sometimes called **acid halides**, are structurally related to carboxylic acids by the replacement of the OH group by a halogen, most often Cl. They are usually named by combining the stems of the common names of the carboxylic acids with the suffix *-yl* and then adding the name of the halide ion. Examples are



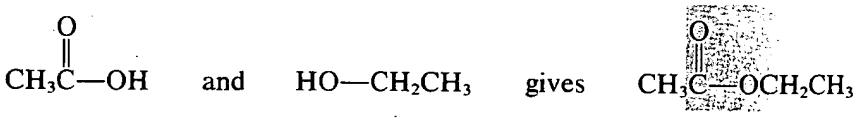
acetyl chloride butyryl fluoride benzoyl chloride

Acid halides are very reactive and have not been observed in nature.

Esters

As we shall see in Section 32-9, one method of forming esters involves acid-catalyzed reaction of an alcohol with a carboxylic acid.

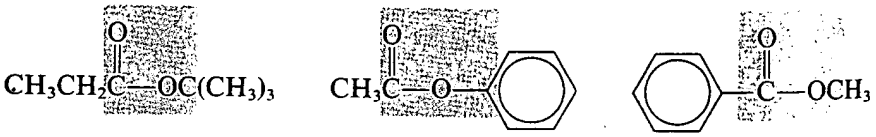
Esters can be thought of as the result of removing one molecule of water from a carboxylic acid and an alcohol. Removing a molecule of water from



acetic acid ethyl alcohol ethyl acetate

Models of ethyl acetate, a simple ester, are shown in Figure 31-18.

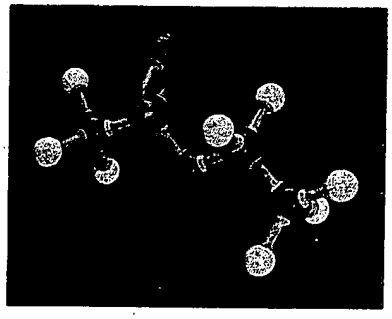
Esters are nearly always called by their common names. These consist of, first, the name of the alkyl group in the alcohol, and then the name of the anion derived from the acid.



tert-butyl propionate phenyl acetate methyl benzoate

Because of their inability to form hydrogen bonds, esters tend to be liquids with boiling points much lower than those of carboxylic acids of similar molecular weight.

Most simple esters are pleasant-smelling substances. They are responsible for the flavors and fragrances of most fruits and flowers and many of the



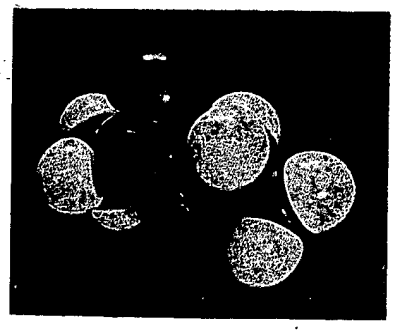


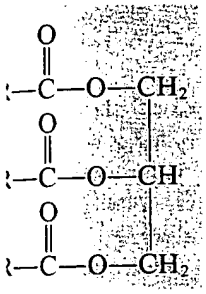
Figure 31-18
Models of ethyl acetate, $CH_3-\overset{\overset{O}{\|}}{C}-O-CH_2CH_3$, an ester. The $CH_3-\overset{\overset{O}{\|}}{C}-$ fragment is derived from acetic acid, the parent acid; the $O-CH_2CH_3$ fragment is derived from ethanol, the parent alcohol.

Table 31-12
Some Common Esters

Ester	Formula	Odor of
n-butyl acetate	$CH_3COOC_4H_9$	bananas
ethyl butyrate	$C_3H_7COOC_2H_5$	pineapples
n-amyl butyrate	$C_3H_7COOC_5H_{11}$	apricots
n-octyl acetate	$CH_3COOC_8H_{17}$	oranges
isoamyl isovalerate	$C_4H_9COOC_5H_{11}$	apples
methyl salicylate	$C_6H_4(OH)(COOCH_3)$	oil of wintergreen
methyl anthranilate	$C_6H_4(NH_2)(COOCH_3)$	grapes

tificial fruit flavorings that are used in cakes, candies, and ice cream (Table 1-12). Esters of low molecular weight are excellent solvents for nonpolar ompounds. Ethyl acetate is an excellent solvent that gives many nail polish movers their characteristic odor.

Fats (solids) and **oils** (liquids) are esters of glycerol and aliphatic acids of igh molecular weight. "Fatty acids" are all organic acids that occur in fats nd oils (as esters). Fats and oils have the general formula



The fatty acid portions, R—C(=O)—, may be saturated or unsaturated. The R's may be the same or different groups.

Fats are solid esters of glycerol and (mostly) saturated acids. Oils are iquid esters that are derived primarily from unsaturated acids and glycerol. The acid portion of a fat usually contains an even number of carbon atoms, often 16 or 18. The acids that occur most frequently in fats and oils are

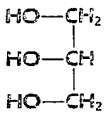
butyric	$CH_3CH_2CH_2COOH$
lauric	$CH_3(CH_2)_{10}COOH$
myristic	$CH_3(CH_2)_{12}COOH$
palmitic	$CH_3(CH_2)_{14}COOH$
stearic	$CH_3(CH_2)_{16}COOH$
oleic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
linolenic	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
ricinoleic	$CH_3(CH_2)_5CHOHCH_2CH{=}CH(CH_2)_7COOH$

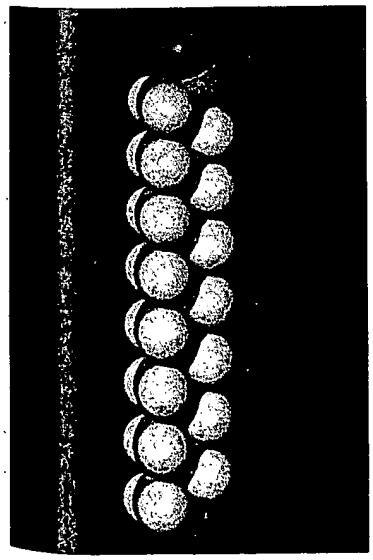
Figure 31-19 is a model of stearic acid, a long-chain saturated fatty acid.

Apple blossoms.

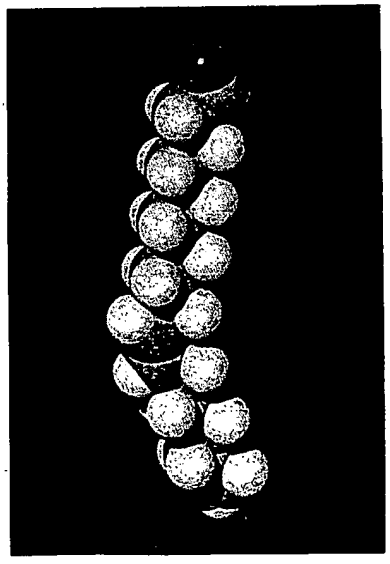
Most natural fatty acids contain even numbers of carbon atoms because they are synthesized in the body from two-carbon acetyl groups.

Glycerol is





(a)

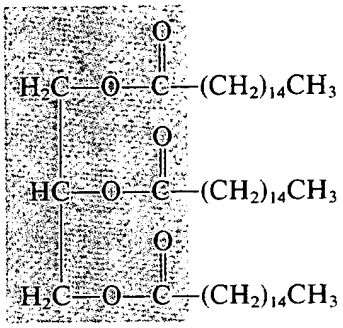


(b)

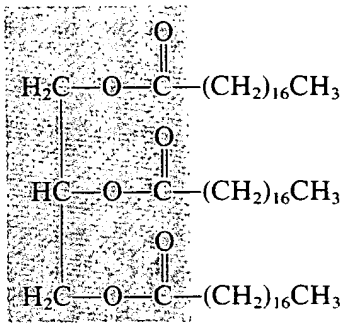
Figure 31-19
Models of long-chain fatty acids. The saturated fatty acids (a) are linear and tend to pack, like sticks of wood, to form solid masses in blood vessels, thereby constricting them. The *trans* unsaturated fatty acids have a slight Z-shaped kink in the chain, but are also essentially linear molecules. By contrast, *cis* unsaturated fatty acids (b) are bent and so do not pack as well as linear structures and do not collect in blood vessels as readily. Many natural vegetable fats contain esters of *cis* unsaturated fatty acids or polyunsaturated fatty acids. Health problems associated with saturated fatty acids can be decreased by eating less animal fat, butter, and lard. Problems due to *trans* fatty acids are reduced by avoiding processed vegetable fats.

Naturally occurring fats and oils are mixtures of many different esters. Milk fat, lard, and tallow are familiar, important fats. Soybean oil, cottonseed oil, linseed oil, palm oil, and coconut oil are examples of important oils.

The triesters of glycerol are called glycerides. *Simple glycerides* are esters in which all three R groups are identical. Two examples are

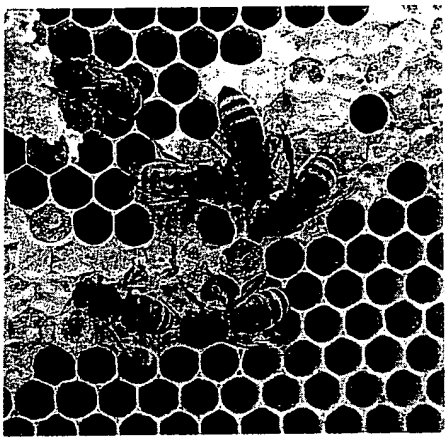


glyceryl tripalmitate (palmitin)



glyceryl tristearate (stearin)

Glycerides are frequently called by their common names, indicated in parentheses in the examples above. The common name is the characteristic stem for the parent acid plus an *-in* ending.



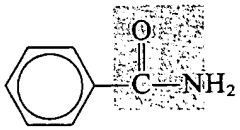
Honeybees produce the wax to build their honeycombs.

Waxes are esters of fatty acids and alcohols other than glycerol. Most are derived from long-chain fatty acids and long-chain monohydric alcohols. Both usually contain even numbers of carbon atoms. Beeswax is largely $C_{15}H_{31}COOC_{30}H_{61}$; carnauba wax contains $C_{25}H_{51}COOC_{30}H_{61}$. Both are esters of myricyl alcohol, $C_{30}H_{61}OH$.

Amides

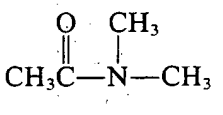
Amides are thought of as derivatives of organic acids and primary or secondary amines. Amides contain the  grouping of atoms. They are named as derivatives of the corresponding carboxylic acids, the suffix *-amide* being substituted for *-ic acid* or *-oic acid* in the name of the parent acid.

acetamide

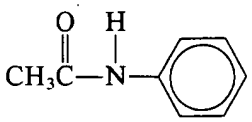


benzamide

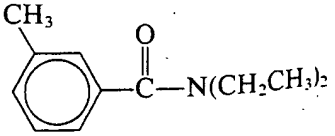
The presence of alkyl or aryl substituents attached to nitrogen is designated by prefixing the letter N and the name of the substituent to the name of the unsubstituted amide.



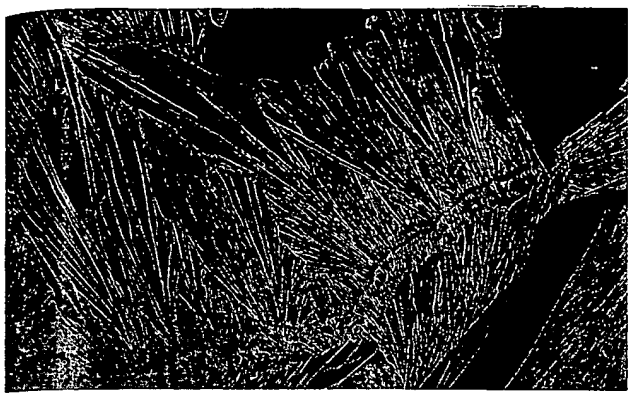
N,N-dimethylacetamide



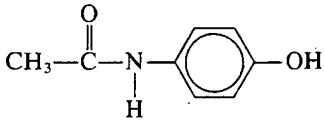
N-phenylacetamide (acetanilide)



N,N-diethyl-*m*-toluamide



Crystals of acetaminophen (Tylenol) viewed under polarized light. The structure of acetaminophen is

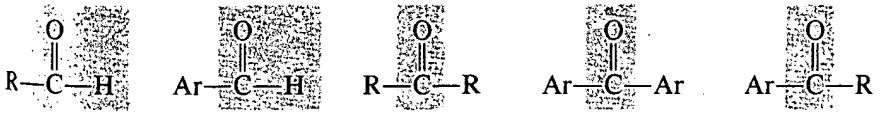


Unsubstituted amides (with the exception of formamide, $HCONH_2$) are crystalline solids at room temperature, with melting and boiling points even higher than those of the carboxylic acids of comparable molecular weight. Dimethylformamide, $HCON(CH_3)_2$, is a good solvent for both polar and nonpolar compounds; it is useful as a reaction medium when such different compounds need to be brought into contact with one another. Acetanilide (sometimes called antifebrin) is the amide of acetic acid and aniline. It is used to treat headaches, neuralgia, and mild fevers. N,N-diethyl-*m*-toluamide, the amide of metatoluic acid and N,N-diethylamine, is the active ingredient in some insect repellents. Proteins are complex amides of high molecular weight. Some synthetic fibers are also polyamides (Section 32-11).

31-14 Aldehydes and Ketones

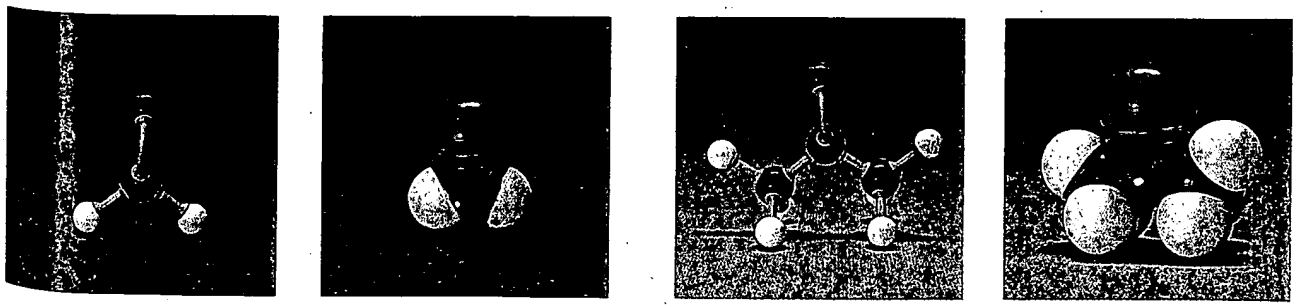
Aldehydes and ketones contain the carbonyl group, $\diagdown C{=}O \diagup$. In **aldehydes**, at least one H atom is bonded to the carbonyl group. **Ketones** have two alkyl or aryl groups bonded to a carbonyl group. Models of formaldehyde (the simplest aldehyde) and acetone (the simplest ketone) are shown in Figure 31-20.

The simplest aldehyde, formaldehyde, H—C(=O)—H, has two H atoms and no alkyl or aryl groups. Other aldehydes have one alkyl or aryl group and one H atom bonded to the carbonyl group.



aliphatic aldehyde | aromatic aldehyde | aliphatic ketone | aromatic ketone | mixed ketone

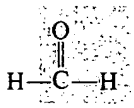
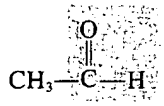
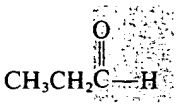
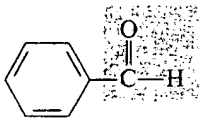
Aldehydes are usually called by their common names. These are derived from the name of the acid with the same number of C atoms (Table 31-13).



(a) (b)

Figure 31-20
(a) Models of formaldehyde, HCHO, the simplest aldehyde. (b) Models of acetone, $CH_3—CO—CH_3$, the simplest ketone.

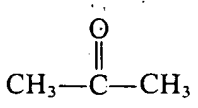
Table 31-13
Properties of Some Simple Aldehydes

Common Name	Formula	Normal bp (°C)
formaldehyde (methanal)		−21
acetaldehyde (ethanal)		20.2
propionaldehyde (propanal)		48.8
benzaldehyde		179.5

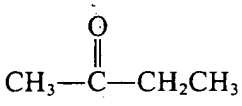
The systematic (IUPAC) name is derived from the name of the parent hydrocarbon. The suffix *-al* is added to the characteristic stem. The carbonyl group takes positional precedence over other substituents.

Formaldehyde has long been used as a disinfectant and as a preservative for biological specimens (including embalming fluid). Its main use is in the production of certain plastics and in binders for plywood. Many important natural substances are aldehydes and ketones. Examples include sex hormones, some vitamins, camphor, and the flavorings extracted from almonds and cinnamon. Aldehydes contain a carbon–oxygen double bond, so they are very reactive compounds. As a result, they are valuable in organic synthesis, particularly in the construction of carbon chains.

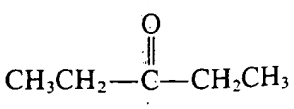
The simplest ketone is called acetone. Other simple, commonly encountered ketones are usually called by their common names. These are derived by naming the alkyl or aryl groups attached to the carbonyl group.



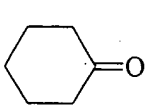
acetone



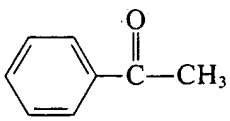
methyl ethyl ketone



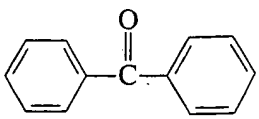
diethyl ketone



cyclohexanone

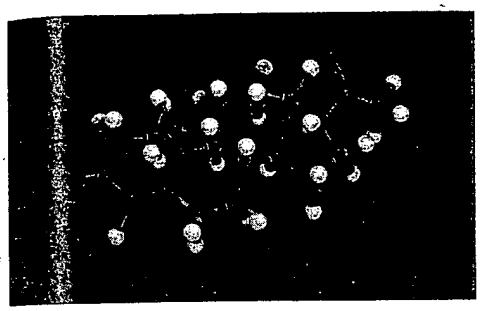


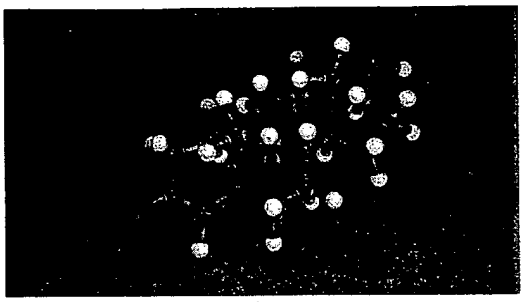
acetophenone
(methyl phenyl ketone)



benzophenone
(diphenyl ketone)

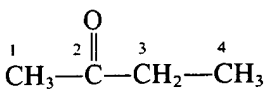
When the benzene ring is a substituent, it is called a phenyl group ($-C_6H_5$).



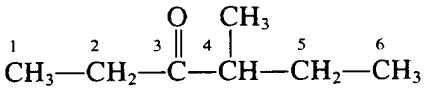


Steroid molecules have similar molecular shapes but different biochemical functions. Progesterone (left), a female sex hormone, and testosterone (right), a male sex hormone. Both are ketones.

The systematic names for ketones are derived from their parent hydrocarbons. The suffix *-one* is added to the characteristic stem.

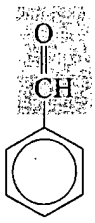


2-butanone

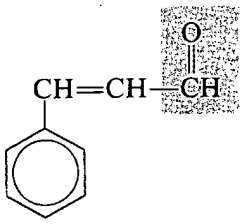


4-methyl-3-hexanone

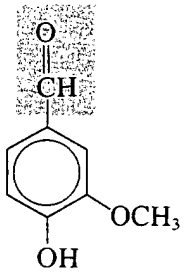
The ketones are excellent solvents. Acetone is very useful because it dissolves most organic compounds yet is completely miscible with water. Acetone is widely used as a solvent in the manufacture of lacquers, paint removers, explosives, plastics, drugs, and disinfectants. Some ketones of high molecular weight are used extensively in blending perfumes. Structures of some naturally occurring aldehydes and ketones are



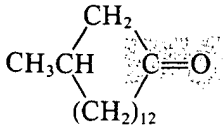
benzaldehyde
(almonds)



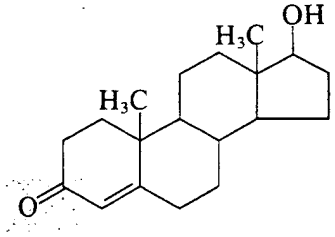
cinnamaldehyde
(cinnamon)



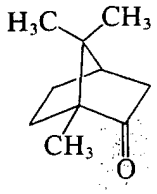
vanillin (vanilla)



muscone
(musk deer, used
in perfumes)



testosterone
(male sex hormone)



camphor

31-15 Summary of Functional Groups

Some important functional groups and the corresponding classes of related compounds are summarized in Figure 31-21.

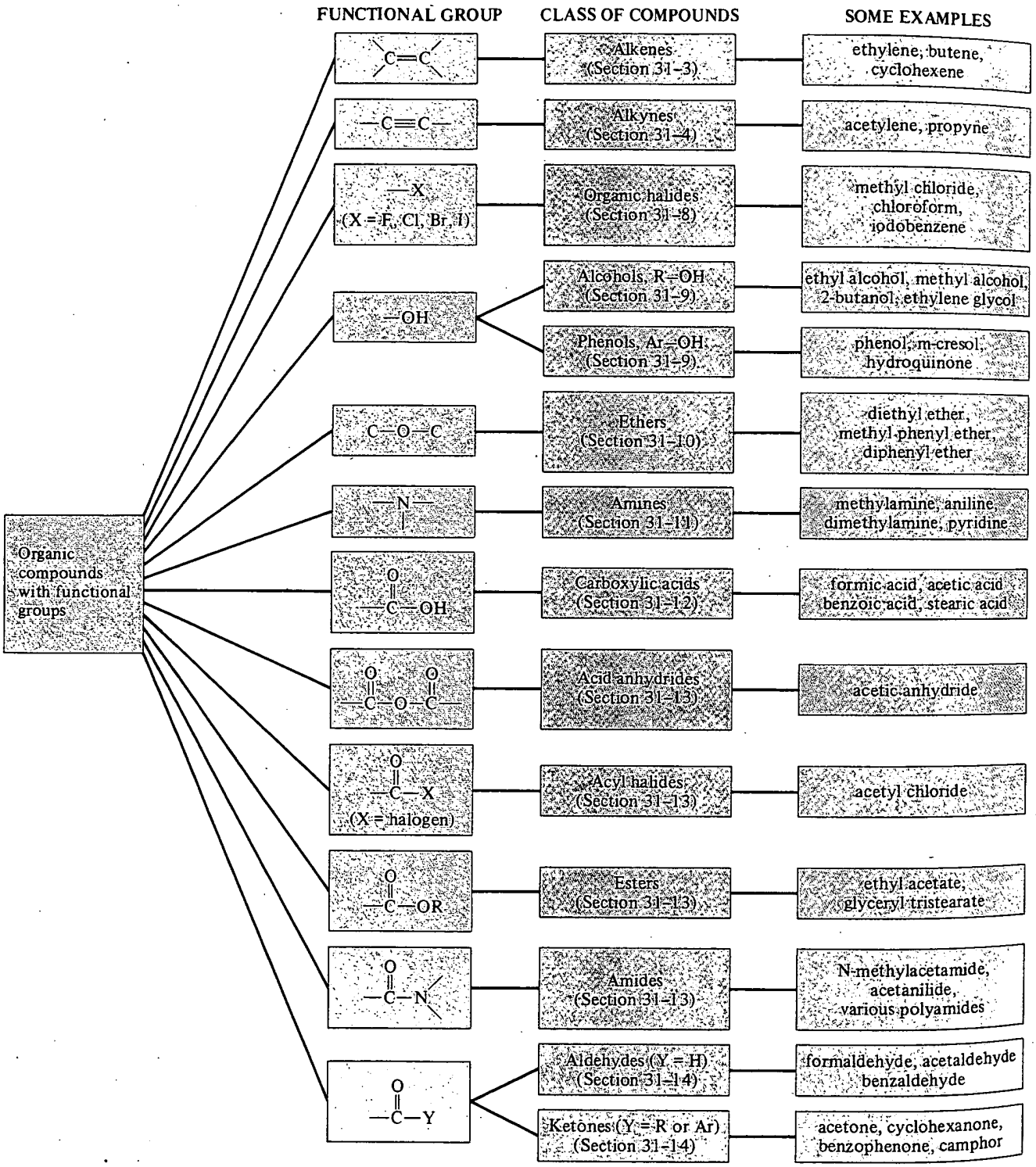


Figure 31-21
Summary of some functional groups and classes of organic compounds.

y Terms

d anhydride A compound produced by dehydration of a carboxylic acid; general formula is $R-C(=O)-O-C(=O)-R$.

id halide See *Acyl halide*.

yl group The group of atoms remaining after removal of an —OH group of a carboxylic acid.

yl halide A compound derived from a carboxylic acid by replacing the —OH group with a halogen (X), usually —Cl; general formula is $R-C(=O)-X$; also called acid halide.

ohol A hydrocarbon derivative containing an —OH group attached to a carbon atom not in an aromatic ring.

lehyde A compound in which an alkyl or aryl group and a hydrogen atom are attached to a carbonyl group; general formula is $R-C(=O)-H$.

phatic hydrocarbons Hydrocarbons that do not contain aromatic (delocalized) rings.

anes See *Saturated hydrocarbons*.

enes (olefins) Unsaturated hydrocarbons that contain one or more carbon–carbon double bonds.

kyl group A group of atoms derived from an alkane by the removal of one hydrogen atom.

kylbenzene A compound containing an alkyl group bonded to a benzene ring.

kynes Unsaturated hydrocarbons that contain one or more carbon–carbon triple bonds.

nide A compound containing the $-C(=O)-N<$ group.

nine A compound that can be considered a derivative of ammonia, in which one or more hydrogens are replaced by alkyl or aryl groups.

nino acid A compound containing both an amino group and a carboxylic acid group.

nino group The $-NH_2$ group.

omatic hydrocarbons Benzene and its derivatives; contain delocalized rings.

yl group The group of atoms remaining after a hydrogen atom is removed from an aromatic system.

rbonyl group The $-C(=O)-$ group.

rboxylic acid A compound containing a $-C(=O)-O-H$ group.

tenation The ability of an element to bond to itself.

nformations Structures of a compound that differ by the extent of rotation about a single bond.

Conjugated double bonds Double bonds that are separated from each other by one single bond, as in C=C—C=C.

Cycloalkanes Cyclic saturated hydrocarbons.

Ester A compound of the general formula $R-C(=O)-O-R'$ where R and R′ may be the same or different, and may be either aliphatic or aromatic.

Ether A compound in which an oxygen atom is bonded to two alkyl or two aryl groups, or one alkyl and one aryl group.

Fat A solid triester of glycerol and (mostly) saturated fatty acids.

Fatty acid An aliphatic acid; many can be obtained from animal fats.

Functional group A group of atoms that represents a potential reaction site in an organic compound.

Geometrical isomers Compounds with different arrangements of groups on the opposite sides of a bond with restricted rotation, such as a double bond or a single bond in a ring; for example, *cis–trans* isomers of certain alkenes.

Glyceride A triester of glycerol.

Heterocyclic amine An amine in which the nitrogen is part of a ring.

Homologous series A series of compounds in which each member differs from the next by a specific number and kind of atoms.

Hydrocarbon A compound that contains only carbon and hydrogen.

Ketone A compound in which a carbonyl group is bound to two alkyl or two aryl groups, or to one alkyl and one aryl group.

Oil A liquid triester of glycerol and unsaturated fatty acids.

Olefins See *Alkenes*.

Organic chemistry The chemistry of substances that contain carbon–hydrogen bonds.

Phenol A hydrocarbon derivative that contains an —OH group bound to an aromatic ring.

Pi bonds A chemical bond formed by the side-to-side overlap of atomic orbitals.

Polyene A compound that contains more than one double bond per molecule.

Polyhydric alcohol An alcohol that contains more than one —OH group.

Primary alcohol An alcohol with no or one R group bonded to the carbon bearing the —OH group.

Primary amine An amine in which one H atom of ammonia has been replaced by an organic group.

Saturated hydrocarbons Hydrocarbons that contain only single bonds. They are also called *alkanes* or *paraffin hydrocarbons*.

Sean D. Monahan

Advanced Organic Chemistry

SECOND EDITION

Part A: Structure and Mechanisms

FRANCIS A. CAREY
and RICHARD J. SUNDBERG

University of Virginia
Charlottesville, Virginia

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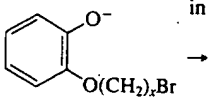
Scheme 3.4. Relative Rates of Ring Closure as a Function of Ring Size

	Reaction	Ring size =	Relative rate 3	4	5	6	7	8
1.[a]	$Br(CH_2)_xCO_2^- \rightarrow$ lactone		8.3×10^{-4}	0.31	90	1	0.0052	6×10^{-5}
2.[b]	$Br(CH_2)_xNH_2 \rightarrow$ cyclic amine		0.07	0.001	100	1	0.002	—
3.[c]	$PhC(=O)(CH_2)_xCl \rightarrow$ nucleophilic participation in solvolysis		—	0.37	36	1	0.13	—
4.[d]	 → cyclic ether formation		—	—	—	1	0.01	4×10^{-4}
5.[e]	$ArSO_2\bar{N}(CH_2)_xCl \rightarrow$ cyclization		17	33	—	1	—	—

a. C. Galli, G. Illuminati, L. Mandolini, and P. Tamborra, *J. Am. Chem. Soc.* **99**, 2591 (1977); L. Mandolini, *J. Am. Chem. Soc.* **100**, 550 (1978).
b. D. F. DeTar and W. Brooks, Jr., *J. Org. Chem.* **43**, 2245 (1978); D. F. DeTar and N. P. Luthra, *J. Am. Chem. Soc.* **102**, 4505 (1980).
c. D. J. Pasto and M. P. Serve, *J. Am. Chem. Soc.* **87**, 1515 (1965).
d. G. Illuminati, L. Mandolini, and B. Masci, *J. Am. Chem. Soc.* **96**, 1422 (1974).
e. R. Bird, A. C. Knipe, and C. J. M. Stirling, *J. Chem. Soc. Perkin Trans. II*, 1215 (1973).

3.9. Relationships between Ring Size and Facility of Ring Closure

Many examples of intramolecular reactions have served to establish a rough correlation between the rate of a reaction and the size of a ring being formed. Although different reaction types exhibit large quantitative differences, so that there are exceptions, the order $5 > 6 > 3 > 7 > 4 > 8$–10 is a rough guide of relative reactivity for many systems. Some quantitative data on typical reactions involving nucleophilic substitution or participation are shown in Scheme 3.4.

The dissection of the energy of activation of typical ring closure reactions usually shows some consistent features. The $\Delta H^{\ddagger}$ for three- and four-membered rings is normally higher than $\Delta H^{\ddagger}$ for the five- and six-membered rings, while $\Delta S^{\ddagger}$ is least negative for the three-membered rings, of comparable magnitude for four-, five- and six-membered rings and then becomes more negative as the ring size increases above seven. The $\Delta H^{\ddagger}$ reflects the strain which develops in the closure of three-membered rings, while the large negative entropy associated with eight-membered and larger rings, reflects the relative improbability of achieving the desired molecular orientation. Because the combination of the two factors is most favorable for five- and six-membered rings, the maximum rate of ring closure is achieved.

Superimposed on this broad relationship between enthalpy and entropy are more variable and individualized structural features, including changes in solvation and the effect of branching on the intervening chain. Most important, however, are

Table 3.11. Classification of Ring-Closure Types[a]

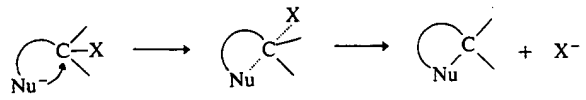
Ring size	Exocyclic bonds			Endocyclic bonds[b]	
	sp (*dig*)	sp^2 (*trig*)	sp^3 (*tet*)	*sp* (*dig*)	sp^2 (*trig*)
3	unfav	fav	fav	fav	fav
4	unfav	fav	fav	fav	unfav
5	fav	fav	fav	fav	unfav
6	fav	fav	fav	fav	fav
7	fav	fav	fav	fav	fav

a. J. E. Baldwin, *J. Chem. Soc., Chem. Commun.*, 734 (1976).
b. The category *endo–tet* also exists but is somewhat rare and is not discussed here.

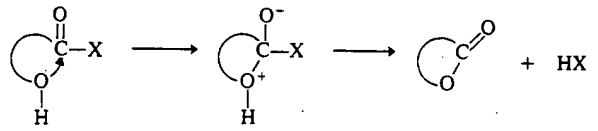
geometric (stereoelectronic) constraints on the transition state for ring closure. There will be a preferred direction of approach in the transition state which will vary depending upon the type of reaction which is involved. While the reactions shown in Scheme 3.3, which are all intramolecular nucleophilic substitutions, reveal a rough general trend $5 > 6 > 3 \sim 7 > 8$, reactions with other mechanisms may exhibit a different kind of relationship. It has been pointed out for example that the formation of cyclopropanes from β-halo carbanions is often *faster* than cyclization of the corresponding γ-halo systems.[95]

A systematic effort to correlate ease of ring closure with the stereoelectronic requirements of the transition state has been discussed by Baldwin and coworkers. They classify ring closures with respect to three factors: (a) ring size, (b) the hybridization at the carbon undergoing reaction, and (c) the relationship of the reacting bond to the forming ring (endocyclic or exocyclic). Consideration of experimental data on ring closure rates reveals certain types are more favorable than others. These relationships are summarized in Table 3.11.

The classifications can be made clear with a few examples. All of the nucleophilic substitutions shown in Scheme 3.3 are of the *exo–tet* classification. The reacting atom is of sp^3 hybridization (tetrahedral) and the reacting bond, that is the bond to the leaving group, is exocyclic to the forming ring. An example of an *exo–trig*

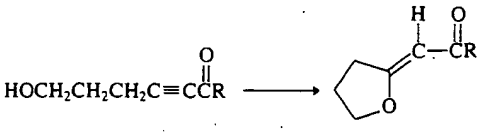


process would be a lactonization of a ω-hydroxycarboxylic acid derivative. An

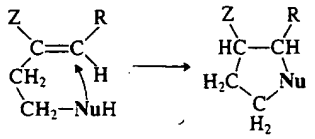


95. C. J. M. Stirling, *J. Chem. Educ.* **50**, 844 (1973).

example of an *exo–dig* process would be the base-catalyzed cyclization of ε-hydroxy-α,β-ynone. Let us focus attention on the unfavorable ring closures. Why, for

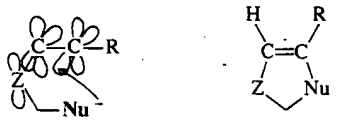


example, should formation of a five-membered ring by an *endo–trig* process be difficult? The answer is provided by consideration of the trajectory of approach of



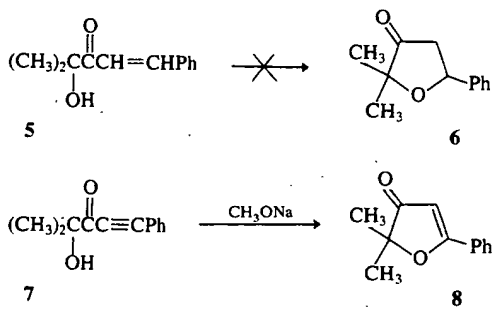
the nucleophile.[96] If Z, for example, is an electron-attracting conjugating group of the type which would be necessary to activate the double bond to nucleophilic attack, the reaction would involve the LUMO of the conjugated system, a π orbital. The nucleophile cannot approach from the plane of this π system, it must

attack from above or below. This would lead to a large distortion from the approximate planarity which characterizes five-membered rings. It is this distortion imposed by stereoelectronic factors which disfavors 5-*endo–trig* cyclization. In contrast, 5-*endo–dig* cyclization is feasible since the acetylenic system provides an orbital which is available with an alternate geometry of approach. As an example of these



relationships, it was found that compound **5** was unreactive toward base-catalyzed cyclization to **6**, even though this compound is a suitable substrate for intermolecular conjugate addition. On the other hand, **7** is readily cyclized to **8** by heating in the presence of sodium methoxide[97]:

96. J. E. Baldwin, *J. Chem. Soc. Chem. Commun.* 738 (1976).
97. J. E. Baldwin, R. C. Thomas, L. I. Kruse, and L. Silberman, *J. Org. Chem.* **42**, 3846 (1977).



5 6

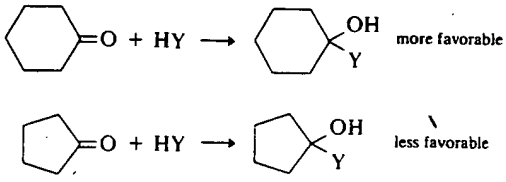
7 8

The terms favored and disfavored imply just that. Other factors will determine the absolute ease of a given ring closure, but these relationships point out the need to recognize the specific stereoelectronic requirements which may be imposed on the transition state in ring closure reactions.

3.10. Torsional Strain and Related Stereoelectronic Effects

Torsional strain refers to the component of total molecular energy which results from nonoptimal arrangement of vicinal bonds. Ethane in the eclipsed conformation is strained by 2.8 kcal/mol relative to the staggered conformation and this is an example of torsional strain. This energy barrier is not primarily the result of van der Waals interactions but arises instead from stereoelectronic effects. The basis for the preference for the staggered conformation was considered in Sections 1.7 and 3.2.

The preference for staggered arrangements around single bonds is general for all alkanes and when geometric constraints enforce an eclipsed arrangement the molecule suffers torsional strain. A case in which torsional effects appear to play a major role is in reactions that involve hybridization changes at ring atoms. A general relationship concerning the relative ease of conversion of carbon atoms in a ring from sp^3 to sp^2 or vice versa has been developed. It has been useful in comparing the reactivity of cyclohexanones with that of cyclopentanones. It has been observed in a number of systems that reactions that convert an sp^2 carbon to an sp^3 carbon in a six-membered ring are more favorable than the corresponding reaction in a five-membered ring.



For example, cyclohexanone is reduced by sodium borohydride 23 times faster than cyclopentanone.[98] The explanation for this difference is believed to lie in the

98. H. C. Brown and K. Ichikawa, *Tetrahedron* **1**, 221 (1957).

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increases in enthalphy. An example is cleavage of ethane into two methyl radicals. In this case a bond of about 79 kcal/mol (330 kJ/mol) is broken, and no new bond is formed to compensate for this enthalpy increase. However, ethane can be cleaved at very high temperatures, which illustrates the principle that *entropy becomes more important as the temperature increases*, as is obvious from the equation $\Delta G = \Delta H - T\Delta S$. The enthalpy term is independent of temperature, while the entropy term is directly proportional to the absolute temperature.

4. An acyclic molecule has more entropy than a similar cyclic molecule because there are more conformations (compare hexane and cyclohexane). Ring opening therefore means a gain in entropy and ring closing a loss.

Kinetic Requirements for Reaction

Just because a reaction has a negative ΔG does not necessarily mean that it will take place in a reasonable period of time. A negative ΔG is a *necessary* but not a *sufficient* condition for a reaction to occur spontaneously. For example, the reaction between H_2 and O_2 to give H_2O has a large negative ΔG, but mixtures of H_2 and O_2 can be kept at room temperature for many centuries without reacting to any significant extent. In order for a reaction to take place, *free energy of activation* ΔG^* must be added.[2] This situation is illustrated in Figure 6.1,[3] which is an energy profile for a one-step reaction without an intermediate. In this type of diagram the horizontal axis (called the *reaction coordinate*)[4] signifies the progression of the reaction. ΔG_f^* is the free energy of activation for the forward reaction. If the reaction shown in Figure 6.1 is reversible, ΔG_r^* must be greater than ΔG_f^*, since it is the sum of ΔG and ΔG_f^*.

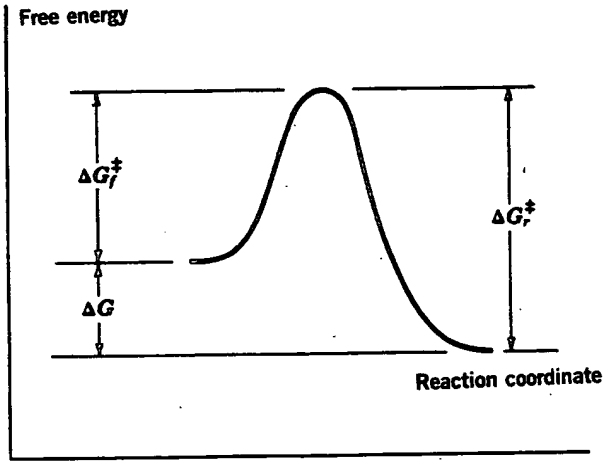


FIGURE 6.1 Free-energy profile of a reaction without an intermediate where the products have a lower free energy than the reactants.

[2]For mixtures of H_2 and O_2 this can be done by striking a match.

[3]Strictly speaking, this is an energy profile for a reaction of the type XY + Z → X + YZ. However, it may be applied, in an approximate way, to other reactions.

[4]For a review of reaction coordinates and structure–energy relationships, see Grunwald *Prog. Phys. Org. Chem.* **1990**, *17*, 55-105.

When a reaction between two or more molecules has progressed to the point corresponding to the top of the curve, the term *transition state* is applied to the positions of the nuclei and electrons. The transition state possesses a definite geometry and charge distribution but has no finite existence; the system passes through it. The system at this point is called an *activated complex*.[5]

In the *transition-state theory*[6] the starting materials and the activated complex are taken to be in equilibrium, the equilibrium constant being designated K^*. According to the theory, all activated complexes go on to product at the same rate (which, though at first sight surprising, is not unreasonable, when we consider that they are all "falling downhill") so that the rate constant (see p. 220) of the reaction depends only on the position of the equilibrium between the starting materials and the activated complex, i.e., on the value of K^*. ΔG^* is related to K^* by

$$\Delta G^* = -2.3RT \log K^*$$

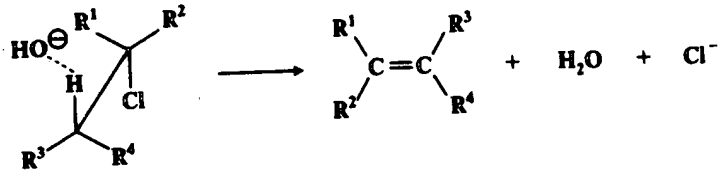
so that a higher value of ΔG^* is associated with a smaller rate constant. The rates of nearly all reactions increase with increasing temperature because the additional energy thus supplied helps the molecules to overcome the activation energy barrier. Some reactions have no free energy of activation at all, meaning that K^* is essentially infinite and that virtually all collisions lead to reaction. Such processes are said to be *diffusion-controlled*.[7]

Like ΔG, ΔG^* is made up of enthalpy and entropy components

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

ΔH^*, the *enthalpy of activation*, is the difference in bond energies, including strain, resonance, and solvation energies, between the starting compounds and the *transition state*. In many reactions bonds have been broken or partially broken by the time the transition state is reached; the energy necessary for this is ΔH^*. It is true that additional energy will be supplied by the formation of new bonds, but if this occurs after the transition state, it can affect only ΔH and not ΔH^*.

Entropy of activation ΔS^*, which is the difference in entropy between the starting compounds and the transition state, becomes important when two reacting molecules must approach each other in a specific orientation in order for the reaction to take place. For example, the reaction between a simple noncyclic alkyl chloride and hydroxide ion to give an alkene (**7-13**) takes place only if, in the transition state, the reactants are oriented as shown.



Not only must the OH^- be near the hydrogen, but the hydrogen must be oriented anti to the chlorine atom.[8] When the two reacting molecules collide, if the OH^- should be near

[5]For a discussion of transition states, see Laidler *J. Chem. Educ.* **1988**, *65*, 540.

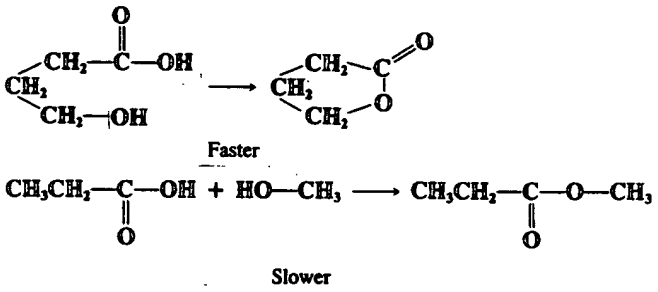
[6]For fuller discussions, see Kreevoy; Truhlar, in Bernasconi, Ref. 25, pt. 1, pp. 13-95; Moore; Pearson *Kinetics and Mechanism*, 3rd ed.; Wiley: New York, 1981, pp. 137-181; Klumpp *Reactivity in Organic Chemistry*; Wiley: New York, 1982; pp. 227-378.

[7]For a monograph on diffusion-controlled reactions, see Rice, *Comprehensive Chemical Kinetics*, Vol. 25 (edited by Bamford; Tipper; Compton); Elsevier: New York, 1985.

[8]As we shall see in Chapter 17, with some molecules elimination is also possible if the hydrogen is oriented syn, instead of anti, to the chlorine atom. Of course, this orientation also requires a considerable loss of entropy.

the chlorine atom or near R^1 or R^2, no reaction can take place. In order for a reaction to occur, the molecules must surrender the freedom they normally have to assume many possible arrangements in space and adopt only that one that leads to reaction. Thus, a considerable loss in entropy is involved, i.e., ΔS^* is negative.

Entropy of activation is also responsible for the difficulty in closing rings[9] larger then six-membered. Consider a ring-closing reaction in which the two groups that must interact are situated on the ends of a ten-carbon chain. In order for reaction to take place, the groups must encounter each other. But a ten-carbon chain has many conformations, and in only a few of these are the ends of the chain near each other. Thus, forming the transition state requires a great loss of entropy.[10] This factor is also present, though less so, in closing rings of six members or less (except three-membered rings), but with rings of this size the entropy loss is less than that of bringing two individual molecules together. For example, a reaction between an OH group and a COOH group in the same molecule to form a lactone with a five- or six-membered ring takes place much faster than the same reaction between a molecule containing an OH group and another containing a COOH group. Though ΔH^* is about the



same, ΔS^* is much less for the cyclic case. However, if the ring to be closed has three or four members, small-angle strain is introduced and the favorable ΔS^* may not be sufficient to overcome the unfavorable ΔH^* change. Table 6.1 shows the relative rate constants for the closing of rings of 3 to 23 members all by the same reaction.[11] Reactions in which the transition state has more disorder than the starring compounds,e.g., the pyrolytic conversion of cyclopropane to propene, have positive ΔS^* values and are thus favored by the entropy effect.

Reactions with intermediates are two-step (or more) processes. In these reactions there is an energy "well." There are two transition states, each with an energy higher than the intermediate (Figure 6.2). The deeper the well, the more stable the intermediate. In Figure 6.2*a*, the second peak is higher than the first. The opposite situation is shown in Figure 6.2*b*. Note that in reactions in which the second peak is higher than the first, the overall ΔG^* is less than the sum of the ΔG^* values for the two steps. Minima in free-energy-profile diagrams (*intermediates*) correspond to real species which have a finite though very short

[9]For discussions of the entropy and enthalpy of ring-closing reactions, see De Tar; Luthra *J. Am. Chem. Soc.* **1980,** *102*, 4505; Mandolini *Bull. Soc. Chim. Fr.* **1988,** 173. For a related discussion, see Menger *Acc. Chem. Res.* **1985,** *18*, 128-134.

[10]For reviews of the cyclization of acyclic molecules, see Nakagaki; Sakuragi; Mutai *J. Phys. Org. Chem.* **1989,** *2*, 187-204; Mandolini *Adv. Phys. Org. Chem.* **1986,** *22*, 1-111. For a review of the cyclization and conformation of hydrocarbon chains, see Winnik *Chem. Rev.* **1981,** *81*, 491-524. For a review of steric and electronic effects in heterolytic ring closures, see Valters *Russ. Chem. Rev.* **1982,** *51*, 788-801.

[11]The values for 4, 5, and 6 are from Mandolini *J. Am.Chem. Soc.* **1978,** *100*, 550; the others are from Galli; Illuminati; Mandolini; Tamborra *J. Am. Chem. Soc.* **1977,** *99*, 2591. See also Illuminati; Mandolini *Acc. Chem. Res.* **1981,** *14*, 95-102. See, however, van der Kerk; Verhoeven; Stirling *J. Chem. Soc., Perkin Trans. 2* **1985,** 1355; Benedetti; Stirling *J. Chem. Soc., Perkin Trans. 2* **1986,** 605.

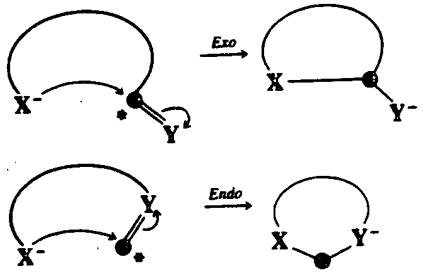
TABLE 6.1 Relative rate constants at 50°C (*Eight-membered ring = 1*) for the reaction $Br(CH_2)_{n-2}CO_2^- \rightarrow (CH_2)_{n-2}\overset{O}{\diagup}C{=}O$, where *n = the ring size*[11]

Ring size	Relative rate
3	21.7
4	5.4×10^3
5	1.5×10^6
6	1.7×10^4
7	97.3
8	1.00
9	1.12
10	3.35
11	8.51
12	10.6
13	32.2
14	41.9
15	45.1
16	52.0
18	51.2
23	60.4

existence. These may be the carbocations, carbanions, free radicals, etc., discussed in Chapter 5 or molecules in which all the atoms have their normal valences. In either case, under the reaction conditions they do not live long (because $\Delta G_2^{\ddagger}$ is small) but rapidly go on to products. Maxima in these curves, however, do not correspond to actual species but only to transition states in which bond breaking and/or bond making have partially taken place. Transition states have only a transient existence with an essentially zero lifetime.[12]

The Baldwin Rules for Ring Closure

In previous sections, we discussed, in a general way, the kinetic and thermodynamic aspects of ring-closure reactions. J. E. Baldwin has supplied a more specific set of rules for certain closings of 3- to 7-membered rings.[13] These rules distinguish two types of ring closure, called



[12]Despite their transient existences, it is possible to study transition states of certain reactions in the gas phase with a technique called laser femtochemistry: Zewall; Bernstein *Chem. Eng. News* **1988,** *66*, No. 45 (Nov. 7), 24-43. For another method, see Collings; Polanyi; Smith; Stolow; Tarr *Phys. Rev. Lett.* **1987,** *59*, 2551.

[13]Baldwin *J. Chem. Soc., Chem. Commun.* **1976,** 734; Baldwin in *Further Perspectives in Organic Chemistry (Ciba Foundation Symposium 53)*; Elsevier North Holland: Amsterdam, 1979, pp. 85-99. See also Baldwin; Thomas; Kruse; Silberman *J. Org. Chem.* **1977,** *42*, 3846; Baldwin; Lusch *Tetrahedron* **1982,** *38*, 2939; Anselme *Tetrahedron Lett.* **1977,** 3615; Fountain; Gerhardt *Tetrahedron Lett.* **1978,** 3985.

FIGURE 6.
energy of
with an int

Exo and
and *Dig*

Rule 1.
(a) 3
(b) 5

Rule 2.
(a) 3
(b) 3
(c) 6

[14]For s
Knochel;

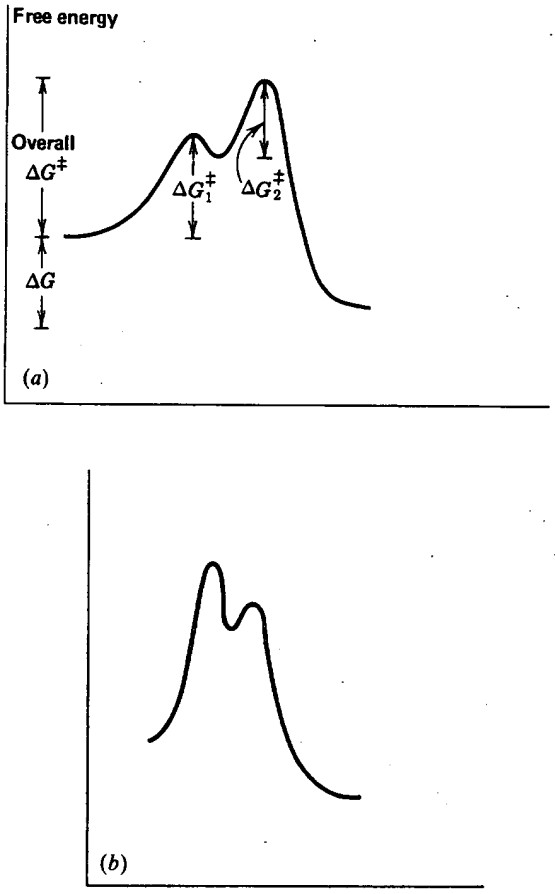


FIGURE 6.2 (*a*) Free-energy profile for a reaction with an intermediate. $\Delta G_1^{\ddagger}$ and $\Delta G_2^{\ddagger}$ are the free energy of activation for the first and second stages, respectively. (*b*) Free-energy profile for a reaction with an intermediate in which the first peak is higher than the second.

Exo and *Endo*, and three kinds of atoms at the starred positions: *Tet* for sp^3, *Trig* for sp^2, and *Dig* for *sp*. The following are Baldwin's rules for closing rings of 3 to 7 members.

Rule 1. Tetrahedral systems
(a) 3 to 7-*Exo-Tet* are all favored processes
(b) 5 to 6-*Endo-Tet* are disfavored

Rule 2. Trigonal systems
(a) 3 to 7-*Exo-Trig* are favored
(b) 3 to 5-*Endo-Trig* are disfavored[14]
(c) 6 to 7-*Endo-Trig* are favored

[14]For some exceptions to the rule in this case, see Trost; Bonk *J. Am. Chem. Soc.* **1985,** *107*, 1778; Auvray; Knochel; Normant *Tetrahedron Lett.* **1985,** *26*, 4455; Torres; Larson *Tetrahedron Lett.* **1986,** *27*, 2223.

Rule 3. Digonal systems
(a) 3 to 4-*Exo-Dig* are disfavored
(b) 5 to 7-*Exo-Dig* are favored
(c) 3 to 7-*Endo-Dig* are favored

"Disfavored" does not mean it cannot be done—only that it is more difficult than the favored cases. These rules are empirical and have a stereochemical basis. The favored pathways are those in which the length and nature of the linking chain enables the terminal atoms to achieve the proper geometries for reaction. The disfavored cases require severe distortion of bond angles and distances. Many cases in the literature are in substantial accord with these rules.

Kinetic and Thermodynamic Control

There are many cases in which a compound under a given set of reaction conditions can undergo competing reactions to give different products:

Figure 6.3 shows a free-energy profile for a reaction in which B is thermodynamically more stable than C (lower ΔG), but C is formed faster (lower $\Delta G^{\ddagger}$). If neither reaction is reversible, C will be formed in larger amount because it is formed faster. The product is said to be *kinetically controlled*. However, if the reactions are reversible, this will not necessarily be the case. If such a process is stopped well before the equilibrium has been established, the reaction will be kinetically controlled since more of the faster-formed product will be present. However, if the reaction is permitted to approach equilibrium, the predominant or even exclusive product will be B. Under these conditions the C that is first formed reverts to A,

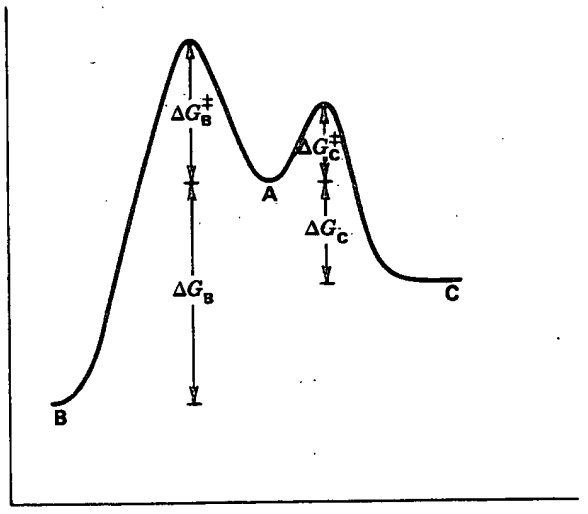


FIGURE 6.3 Free-energy profile illustrating kinetic versus thermodynamic control of product. The starting compound (A) can react to give either B or C.

byterian Hospital, and also the secretarial assistance of Geneva Jackson.

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Urea Dependence of Thiol-Disulfide Equilibria in Thioredoxin: Confirmation of the Linkage Relationship and a Sensitive Assay for Structure†

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ABSTRACT: Thioredoxin contains a single disulfide bond that can be reduced without perturbing significantly the structure of the enzyme. Upon reduction of the disulfide, protein stability decreases. We have experimentally tested the expected linkage relationship between disulfide bond formation and protein stability for thioredoxin. In order to do this, it is necessary to measure the equilibrium constant for disulfide bond formation in both the folded and unfolded states of the protein. Using glutathione as a reference species, we have measured the equilibrium constant for forming the disulfide bond (effective concentration) in thioredoxin as a function of urea concentration. As a control, we show that urea per se does not interfere with our measurements of thiol-disulfide equilibrium constants. Comparison of the values obtained for disulfide bond formation in the folded and unfolded states with the free energies for unfolding oxidized and reduced thioredoxin using circular dichroism confirms the expected linkage relationship. The urea dependence of thiol-disulfide equilibria provides a sensitive assay for folded structure in peptides or proteins. The method should also be useful to evaluate the stabilizing or destabilizing effect of natural or genetically engineered disulfides in proteins. In future work, the effects of amino acid substitutions on disulfide bond formation could be evaluated individually in the native and unfolded states of a protein.

A useful way to think about thermodynamic linkage relationships in protein stability is to consider the effective concentrations of specific interactions in the protein (Creighton, 1983). Effective concentrations represent a ratio of equilibrium or rate constants for otherwise identical intra- and intermolecular reactions. The concept of effective concentrations, used to explain the chelate effect in inorganic chemistry (Schwarzenbach, 1952; Adamson, 1954), is recognized as a useful concept in enzymology (Page & Jencks, 1971). Ef-

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fective concentrations often exceed the concentrations of molecules in liquids or solids. This is understood in terms of the loss of rotational and translational entropy when unimolecular interactions are compared with their bimolecular counterparts (Page & Jencks, 1971).

Because it involves a reversible covalent change, the disulfide is the only interaction found in proteins for which an effective concentration (C_{eff}) can be measured directly and specifically. With glutathione as a reference thiol (Creighton, 1984), C_{eff} is the ratio of the intramolecular equilibrium constant for disulfide bond formation in a peptide or protein to the intermolecular equilibrium constant for forming a disulfide between two molecules of reduced glutathione (Creighton, 1983). Expressing both equilibria (eq 1a and 1b) as half-reactions:

$$P_{SH}^{SH} \overset{K_{intra}}{\rightleftharpoons} P_S^S + 2H^+ + 2e^- \quad (1a)$$

$$2GSH \overset{K_{inter}}{\rightleftharpoons} GSSG + 2H^+ + 2e^- \quad (1b)$$

$$C_{eff} = \frac{K_{intra}}{K_{inter}} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} \quad (1c)$$

where P_{SH}^{SH} and P_S^S refer to the reduced and oxidized forms of the polypeptide and GSH and GSSG refer to reduced and oxidized glutathione. C_{eff} has units of concentration since a unimolecular reaction is being compared to a bimolecular one. In these equations, and subsequently, a concentration of thiols is meant to include the thiolate species [cf. Houk et al. (1987)]. C_{eff} is an empirical parameter, measured relative to a standard species (in this case, glutathione) at a given pH and set of conditions. Experimentally, C_{eff} can be measured in a redox equilibrium mixture of peptide and glutathione:

$$P_{SH}^{SH} + GSSG \rightleftharpoons P_S^S + 2GSH \quad (2a)$$

$$K_{eq} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} = C_{eff} \quad (2b)$$

For convenience, the concentrations of GSH and GSSG are much larger than that of the protein or peptide so that the redox potential of the solution is fixed (Snyder, 1987). The amount of oxidized and reduced protein or peptide is quantitated by HPLC, and C_{eff} is calculated from eq 2b. The reaction between protein and glutathione proceeds through mixed disulfides. Although the mixed disulfide species do not enter into the relevant equilibrium constant (eq 2b), they must be distinguished from other species in the separation.

The ratio of C_{eff} in the folded (N) and unfolded (U) states of a polypeptide gives, by linkage, the difference in free energy for unfolding with or without the disulfide bond:

$$\begin{array}{ccc} N_{SH}^{SH} & \overset{K_1}{\rightleftharpoons} & N_S^S \\ \Big\downarrow{\scriptstyle K_3} & & \Big\downarrow{\scriptstyle K_4} \\ U_{SH}^{SH} & \overset{K_2}{\rightleftharpoons} & U_S^S \end{array} \quad (3a)$$

$$C_{eff}^N/C_{eff}^U = K_1/K_2 = K_3/K_4 \quad (3b)$$

where C_{eff}^N and C_{eff}^U represent C_{eff} of folded and unfolded states of a polypeptide, respectively.

In this paper, we demonstrate that C_{eff}^U can be measured in concentrated urea solutions: the principal concern is that the urea per se will affect measurements of C_{eff}. We show that the measured value of C_{eff} in a model "random-coil" peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2], called $[Gly]_6$, is independent of urea concentration from 0 to 7 M urea. Changes in C_{eff} with [urea] in proteins or peptides are thus likely to represent unfolding and/or destabilization of structure.

Using *Escherichia coli* thioredoxin, we also show that the effective concentration for the single disulfide in the protein decreases in a sigmoidal fashion as urea is added. Then, we test quantitatively the linkage relationship (eq 3b) by comparing the ratio of C_{eff}^N:C_{eff}^U obtained at low and high urea concentrations, respectively, to the ratio of K_3:K_4 obtained by using traditional measurements of the unfolding equilibrium in the absence or presence of the disulfide bond.

The urea titration method used here provides a sensitive probe for folding and stability in polypeptides that can form a disulfide bond. The method can also be used to evaluate the stabilizing or destabilizing effect of a natural or genetically engineered disulfide [cf. Villafranca et al. (1983), Wells and Powers (1986), Schultz et al. (1987), and Wetzel et al. (1988)] and to evaluate the effects of amino acid substitutions on disulfide bond formation in the native and unfolded states of a protein.

Materials and Methods

The model random-coil peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2] was synthesized on an Applied Biosystems Model 430A peptide synthesizer using standard reaction cycles. A *p*-methylbenzhydrylamine resin was used to give the C-terminal amide, and the amino terminus was blocked by acetylation. The peptide was cleaved from the resin with trifluoromethanesulfonic acid (Yajima & Fujii, 1983; Tam et al., 1986). The cleaved peptide was desalted in the reduced form on a Sephadex G-10 column in dilute acetic acid. It was then purified by reverse-phase HPLC on a Vydac C18 column, using a mobile phase composed of acetonitrile, water, and trifluoroacetic acid.

E. coli thioredoxin was purchased from Chemical Dynamics Corp. and purified by reverse-phase HPLC before use. GSH and GSSG were obtained from Sigma and used without further purification. The values of C_{eff} obtained did not depend, within experimental error, on the concentration of GSH over a 3.4-fold range or on the concentration of GSSG over a 2.6-fold range. The redox potential depends on the ratio of [GSSG] to $[GSH]^2$, so the independence of C_{eff} on the concentrations of the reagents used indicates that any impurities are not significant for these measurements. All other chemicals were of reagent grade or better.

The HPLC assay used to determine C_{eff} in peptides or proteins is as follows. The peptide (or protein) is incubated with a mixture of GSH and GSSG in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 8.7. For measurements of C_{eff} in urea solutions, the same conditions are used and the measured pH is adjusted to 8.7 after addition of urea. The concentrations of GSH and GSSG in the reaction mixture are at least 50-fold higher than that of the peptide, in order to fix the redox potential of the solution. A low concentration of peptide ($\sim$50 μM) is used to prevent dimer formation, and the reaction is performed under argon to prevent air oxidation. The reaction is allowed to proceed to equilibrium, as judged by lack of a time dependence for C_{eff} (generally checked after 1–2 h of incubation). After equilibration, the reaction mixture is loaded directly onto a reverse-phase column, previously equilibrated with acidic solvents. Alternatively, the reaction mixture is quenched with HCl (to pH 2) and then loaded onto the column. The reduced and oxidized forms of the peptide (or protein) are detected by absorbance at 229 nm, and the peaks are quantitated by integration. Corrections are made for differences in the relative extinction coefficient at 229 nm for the reduced and oxidized forms, which are determined by injecting the same amount of peptide (or protein) in the reduced or oxidized state.

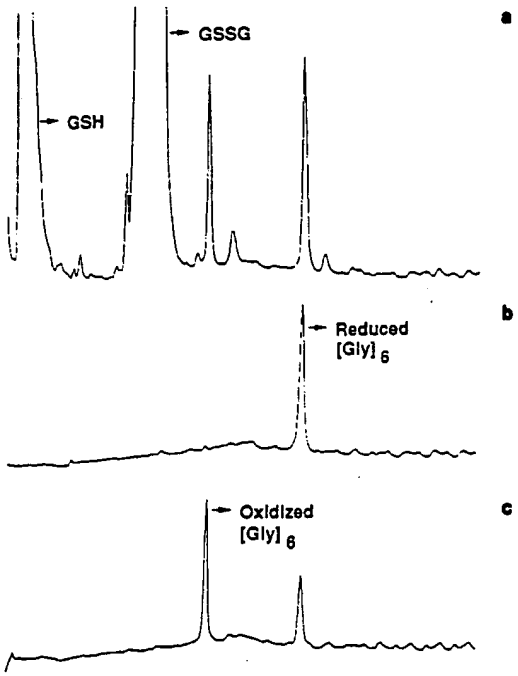

FIGURE 1: Separation of oxidized and reduced forms of a peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2] by reverse-phase HPLC. Solvent A is 0.1% TFA in water, and solvent B is 70% acetonitrile, 30% water, and 0.1% TFA. The gradient is from 100% to 97% solvent A in 72 min. (a) Chromatogram of the peptide in a C_{eff} reaction mixture, which contained 0.1 M Tris, 0.2 M KCl, 1 mM EDTA, 21.6 mM GSH, and 3.6 mM GSSG, pH 8.7. (b) Chromatogram of the reduced peptide obtained by incubation with DTT. (c) Chromatogram of the peptide after air oxidation for 5.5 h at room temperature in 0.28 M Tris, pH 8.7.

As a check on the acid quench, the free thiols of reduced thioredoxin were also quenched with iodoacetamide. The final concentration was between 0.25 and 0.68 M iodoacetamide in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 8.7. After incubation at room temperature for 1–2 min, the solution pH was decreased to 2 by adding HCl and then analyzed by HPLC. HPLC experiments were carried out to ensure that the iodoacetamide quench was not modifying the protein except at thiol groups.

The stability of thioredoxin to unfolding by guanidine hydrochloride (Gdn-HCl) was measured by circular dichroism analysis at 220 nm (Kelley et al., 1987). An Aviv 60DS spectropolarimeter was used with a thermostated cell having a 1-mm path length. Spectra were measured at 23 °C in a solution of 0.1 M Tris-HCl, 0.2 M KCl, and 1 mM EDTA, containing different concentrations of Gdn-HCl (Schwarz-Mann Ultrapure). The pH of the solution was adjusted to 8.7 after addition of Gdn-HCl. Spectra were accumulated from 400 to 210 nm, and the buffer spectrum was subtracted. Solutions of reduced thioredoxin also contained a 10-fold molar excess of reduced DTT.

Results

A representative HPLC chromatogram of a reaction mixture for measuring C_{eff} in the peptide $[Gly]_6$ is shown in Figure 1a. GSH and GSSG elute early in the gradient. The reduced peptide (P_{SH}^{SH}) peak was identified by reducing the peptide with 0.1 M DTT (Figure 1b). The oxidized peptide peak was identified by using a dilute (~50 μM) sample of peptide that was allowed to oxidize at pH 8.7 in the presence of air (Figure 1c). Mass spectrometry analysis confirmed assignments of these peaks.

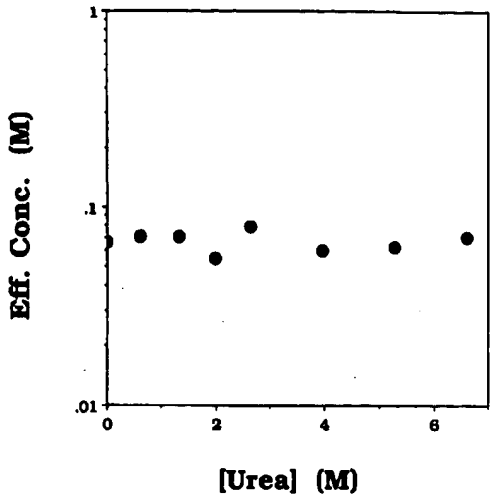

FIGURE 2: Urea dependence of C_{eff} in the peptide Ac-Cys-$(Gly)_6$-Cys-NH_2. C_{eff} was measured in a buffer of 0.1 M Tris, 0.2 M KCl, 1 mM EDTA, and different concentrations of urea at pH 8.7. The measurement was done at room temperature (23 °C). The concentrations of GSSG and GSH were 3.6 and 21.6 mM, respectively. The concentration of peptide was approximately 50 μM.

The relative amounts of P_{SH}^{SH} and P_S^S in the equilibrium mixture are determined by integration of the HPLC peak areas, after correction for differences in extinction coefficients at 229 nm. In the case of $[Gly]_6$, the ratio of the extinction coefficients of P_S^S to P_{SH}^{SH} at 229 nm is 1.5. This difference probably results from the absorbance of the disulfide bond at 229 nm. Knowing the concentrations of GSH, GSSG, P_{SH}^{SH}, and P_S^S, C_{eff} is obtained from eq 2b.

In eq 3b, the ratio between K_1 and K_2 can be obtained by measuring C_{eff} in the folded and unfolded states, which is proportional to K_3 and K_4, respectively. In order to do so, it is necessary to find conditions in which C_{eff} of the unfolded state can be measured accurately. We decided to investigate urea as a denaturant since there is little effect of urea on the pK_a of thiols (Creighton, 1977). The model random-coil peptide, $[Gly]_6$, was used. As shown in Figure 2, C_{eff} for $[Gly]_6$ is about 60 mM, and there is little dependence of C_{eff} on [urea]. This indicates that urea per se is not affecting our measurements of C_{eff}.

E. coli thioredoxin is a relatively small enzyme of 108 amino acids containing a single disulfide bond in the active site (Holmgren et al., 1975). It has been shown that there is little change in the conformation of the protein upon reduction of the disulfide bond (Stryer et al., 1967; Holmgren & Roberts, 1976; Hiraoki et al., 1988).

Figure 3 depicts C_{eff} in thioredoxin as a function of urea concentration. In the absence of urea, C_{eff} is 10 M, indicating a high propensity for the cysteines to form a disulfide in the native protein. As the concentration of urea is increased, C_{eff} decreases to a value of 26 mM, indicating that the propensity to form a disulfide in the unfolded protein is approximately 400 times lower than that in the folded protein. C_{eff} remains constant between 7.7 and 9 M urea (Figure 3), which supports our previous conclusion that urea per se is not affecting our measurements of C_{eff}. The values of C_{eff} shown in Figure 3 are independent of the ratio of GSSG and GSH used in the redox buffer, as described in the preceding section.

Because free thiols are apt to undergo oxidation and thiol–disulfide exchange reactions, a good quench is necessary for measuring C_{eff}. The commonly used quench reagents are acid, iodoacetamide, or iodoacetate. Quenching with acid is

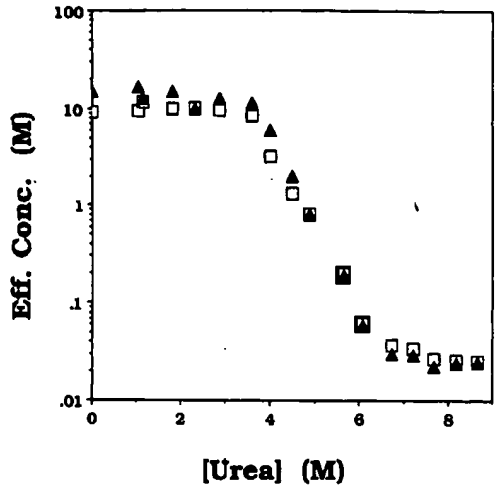

FIGURE 3: Urea dependence of C_{eff} in *E. coli* thioredoxin. C_{eff} were measured at room temperature (23 °C) in the same buffer as described in Figure 2. The concentration of GSSG was 0.56 mM and that of GSH from 3.3 to 94 mM. The concentration of protein was 5–7 μM. (□) Results obtained when the reaction mixture was quenched by acid to pH 2. (▲) Results obtained when the reaction mixture was quenched with iodoacetamide.

very fast, with a rate constant greater than 1×10^9 s^{-1} M^{-1} (Eigen, 1964). It also produces only a small change in the thiol groups as compared to other quenching reagents. The drawback of the acid quench is that it is not irreversible. Quenching with iodoacetate or iodoacetamide is an irreversible reaction. The reaction, however, is relatively slow and thiol–disulfide exchange can occur during quenching. Iodoacetamide or iodoacetate (0.1 M) has been used in studies of BPTI (Creighton, 1974a,b) and an immunoglobulin light chain fragment (Goto & Hamaguchi, 1981). Synder (1987), in studying a soybean trypsin inhibitor fragment that contains two cysteines, found that 1/7 of the thiols convert to disulfides during quenching with 0.1 M iodoacetamide or iodoacetate at pH 7. Creighton showed that acid, iodoacetate, and iodoacetamide give the same one-disulfide intermediates for BPTI but found that the two-disulfide intermediates trapped by acid rearrange intramolecularly (Creighton, 1974a,b, 1984).

We generally used an acid quench, since it is fast and easy. We have compared the results of C_{eff} measurements obtained with an iodoacetamide quench and an acid quench. Quenching with iodoacetamide at high concentrations (≥0.25 M) for 1–2 min at pH 8.7 converts reduced thioredoxin completely to the carboxamidomethylated protein. Figure 3 shows that results obtained with an iodoacetamide quench are comparable to those obtained with an acid quench.

As indicated in eq 3, the finding that C_{eff} in native thioredoxin is higher than that in unfolded thioredoxin is consistent with previous reports that oxidized thioredoxin is more stable than the reduced protein (Kelley et al., 1987). The ratio of C_{eff} for native and unfolded thioredoxin (C_{eff}^N and C_{eff}^U) should be the same as the ratio of equilibrium constants for unfolding reduced and oxidized thioredoxin (K_3 and K_4, respectively, in eq 3). This linkage relationship was tested by measuring K_3 and K_4 independently, in the same conditions used for the C_{eff} measurements. Circular dichroism was used to monitor the Gdn-HCl-induced unfolding of reduced and oxidized thioredoxin. Figure 4a shows that, as predicted, reduced thioredoxin is less stable than the oxidized form. Linear extrapolations (Schellman, 1978; Pace, 1986) of the unfolding free energies were used to estimate the free energy of unfolding reduced and oxidized thioredoxin in the absence of Gdn-HCl,

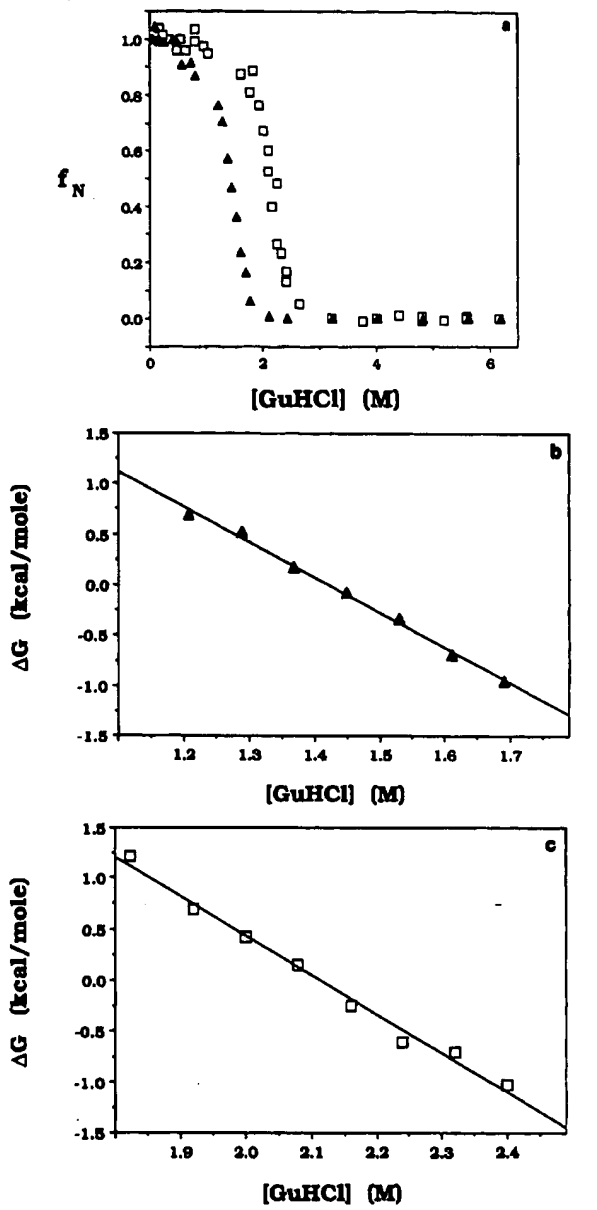

FIGURE 4: (a) Equilibrium unfolding transition for thioredoxin in the presence (□) and absence (▲) of the disulfide bond. (b) Linear extrapolation of the unfolding free energy for reduced thioredoxin showing data within the transition region. The extrapolation gives a $\Delta G°$ of 5.0 kcal/mol for unfolding reduced thioredoxin. (c) Linear extrapolation of the data for oxidized thioredoxin. The extrapolation gives a $\Delta G°$ of 8.1 kcal/mol for unfolding oxidized thioredoxin. Conditions as in Figure 2, except that Gdn-HCl was used instead of urea. Experiments with reduced thioredoxin were performed in the presence of a 10-fold molar excess of DTT.

as shown in panels b and c of Figure 4. The results give unfolding free energies of 5.0 and 8.1 kcal/mol for the reduced and oxidized species, respectively. The difference in stability ($\Delta\Delta G$) is 3.1 ± 0.6 kcal/mol, in good agreement with the predicted $\Delta\Delta G$ of 3.5 ± 0.3 kcal/mol from the ratio of C_{eff}^N and C_{eff}^U.

Discussion

Linkage between Disulfide Bond Formation and Protein Stability. Our results show that the thermodynamic cycle shown in eq 3b can be experimentally confirmed in the case of thioredoxin. This suggests that the two-state approximations

indicated in eq 3b are reasonable descriptions of the linkage between protein stability and disulfide bond formation. Because there appears to be little intrinsic effect of [urea] on disulfide bond stability, the assay described here circumvents the base-line extrapolation problems normally associated with evaluating K_3 and K_4 by traditional methods (Schellman, 1987; Pace, 1986).

Thus, measurements of the urea dependence of disulfide bond stability, similar to those used here, are intrinsically more accurate than measurements of conformational stability and involve fewer assumptions. As described below, it may also be possible to use such measurements to investigate the effects of amino acid substitutions on disulfide bond formation in the native and denatured states of a protein or peptide.

Effective Concentration Assay for Structure. In the structure assay used here, the effective concentration between two thiols in the peptide or protein is measured at varying concentrations of a denaturant (urea). Unfolding of the structure by urea is likely to change C_{eff}: the extent of change in C_{eff} is related by linkage to the effect of the disulfide bond on stability (eq 3b). The assay described here can be used as a sensitive test for structure in proteins, peptides, and protein-folding intermediates.

An assumption of the assay is that urea does not have any intrinsic effects on measurements of C_{eff}. This assumption is supported by our finding that C_{eff} in the model random-coil peptide ($[Gly]_6$) is independent of urea concentration (Figure 2). In addition, C_{eff} in thioredoxin is independent of urea concentration at high [urea] where the protein is unfolded (Figure 3).

The sensitivity of the assay depends on the difference between C_{eff} in the folded and unfolded conformations. In the case of thioredoxin this difference is approximately 400-fold. Assuming that one can detect a 2-fold change in C_{eff} with confidence (this seems conservative given the scatter in the data shown in Figures 2 and 3), a population containing 0.3% folded molecules could be detected for thioredoxin. In other proteins with a larger difference between C_{eff}^{N} and C_{eff}^{U}, the sensitivity of the assay can be orders of magnitude greater. For example, C_{eff} for the three disulfides in bovine pancreatic trypsin inhibitor has been estimated to be 200, 2×10^3, and 1×10^5 M, while C_{eff} for these disulfide bonds in unfolded BPTI has been estimated to be approximately 0.05 M (Creighton & Goldenberg, 1984; Creighton, 1988).

In addition to the high sensitivity of the assay, it is also precise over a wide range of C_{eff} values. This is because the redox potential of the solution can be varied by adjusting the ratio of [GSH]:[GSSG] so that the oxidized and reduced forms of the peptide or protein are present in approximately equal amounts (i.e., where quantitation of the relative amounts of the two species is most precise). Since the redox potential of the solution varies with the square of [GSH] but linearly with [GSSG], it is possible to accommodate a large range of C_{eff} values. Measurements of large C_{eff} values, however, will be limited by the solubility of GSH (~0.4 M at pH 8.7). In such cases, other disulfide reagents with greater reducing potential (e.g., DTT) could be used instead of GSH. C_{eff} for DTT, relative to glutathione, has been estimated to be 1200 M at pH 8.7 in the same conditions as used here (Creighton & Goldenberg, 1984; Creighton, 1986), so it should be possible to compare results obtained with DTT to those obtained with GSH.

It should be noted that the assay will also work if C_{eff} in the native state is *lower* than that in the unfolded state. In such a case, structure would be detected by an increase in C_{eff} as urea is added. The linkage relationship in eq 3 indicates that such a result would identify a disulfide bond that has a destabilizing effect on the protein or peptide.

The major limitation of the assay described here is that it is best suited for peptides or proteins that contain a single disulfide. In theory it is possible to extend the assay to include systems containing multiple disulfides, but it is simpler to work with single disulfide containing species. The assay will also not detect structure if C_{eff} in the native state is the same as that in the unfolded state. This latter situation is expected to be rare, although as pointed out earlier, it is the difference between the two C_{eff} values that determines the sensitivity of the assay.

From a practical point of view, the assay is advantageous because little material is required. A complete urea dependence study will typically require less than 1 mg of peptide or protein, which can be recovered for reuse. Air oxidation must be avoided, and we find it necessary to work in an argon atmosphere and to purge solutions with argon before use. We have found that reverse-phase HPLC gives good resolution and recovery for reduced and oxidized thioredoxin as well as many other peptides. If adequate recovery or separation is not obtained by HPLC, other methods such as electrophoresis might be used [cf. Creighton (1974a), Goto and Hamaguchi (1981), and Wells and Powers (1986)].

The reaction between protein and glutathione proceeds through mixed disulfide species. Mixed disulfides do not need to be quantitated since they are not part of the equilibrium being considered (eq 2) and because GSH and GSSG are in vast excess (i.e., the presence of mixed disulfides does not alter the redox potential of the solution). It is important, however, to separate the mixed disulfide species from the reduced and oxidized species of the peptide or protein. We check for incomplete separation using a very slow gradient (<0.2% acetonitrile increase/min).

Problems of Quenching. Although quenching is necessary to stop the thiol–disulfide reaction, it is difficult to find an ideal method. The following problems are often encountered: (i) The quenching reaction is not irreversible or the quenched product is not stable; therefore, the spectrum of species changes with time. (ii) The rate of quenching may be different for different species. Thus, some species may be trapped while others may interconvert.

Our results with thioredoxin indicate that both acid and iodoacetamide quenching give comparable results. Thus, both problems mentioned above are probably negligible, provided that the concentration of iodoacetamide used is high (≥0.25 M). At high concentrations of iodoacetamide, however, side reactions can become a problem. For thioredoxin, we find that exposure to iodoacetamide gradually converts the protein to another species, indicating that there are reactive groups other than thiols that can be modified. Our quench conditions, 0.25–0.68 M iodoacetamide for 1–2 min, were chosen to ensure effective quenching of thiols with negligible side reactions.

Effects of Mutation on the Native and Denatured States of a Protein. In theory, amino acid replacements can affect the stability of proteins by changing the free energy of the native state, denatured state, or both. Traditional thermodynamic measurements determine the *difference* in free energy between the native and denatured states, so it is difficult to evaluate how an amino acid replacement is altering protein stability. Most structural studies of amino acid replacements focus on the native state; it has been difficult to study the effects of amino acid replacements on the structure of the unfolded state.

It may be possible to use disulfide bonds to probe the effects of mutations in the native and denatured states of a protein. This would involve determining values of C_{eff}^{N} and C_{eff}^{U} (eq 3) in two proteins that differ by a single amino acid. These considerations have been discussed by Goldenberg and Creighton (1984) and Alber (1988). Our finding that urea does not have intrinsic effects on measurements of C_{eff} permits determination of C_{eff}^{U} directly (eq 3). Thus, it should now be possible to determine the effects of amino acid substitutions on an interaction (i.e., formation of a specific disulfide bond) in both the native and unfolded states of proteins. A caveat is that this approach introduces two new states to be evaluated; instead of considering the native and denatured states alone, one needs to consider the reduced and oxidized forms of each state.

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Kinetics and Equilibria of the Formation and Reduction of the Disulfide Bonds in Arginine-Vasopressin and Oxytocin by Thiol/Disulfide Interchange with Glutathione and Cysteine

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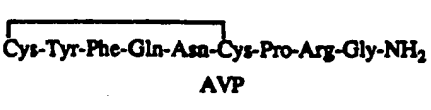
Rate and equilibrium constants are reported for reduction of the disulfide bonds in the neurohypophyseal peptide hormones oxytocin (OT) and arginine-vasopressin (AVP) by thiol/disulfide interchange with glutathione (GSH) and cysteine (CySH) and for formation of the disulfide bonds by thiol/disulfide interchange with oxidized glutathione (GSSG) and cystine (CySSCy). The reactions take place in two steps. In the first step of the reduction reactions, AVP and OT react with GSH and CySH to form peptide-GSH and peptide-CySH mixed disulfides, which in turn react with another molecule of GSH or CySH to give the reduced dithiol form of the peptide and GSSG or CySSCy. Analysis of the forward and reverse rate constants indicates that which step is rate determining depends on the concentration of GSH or CySH. At physiological concentrations of GSH and CySH, intramolecular thiol/disulfide interchange in the mixed disulfides to reform the native disulfide bonds is faster than reaction with another molecule of GSH or CySH, even though intramolecular thiol/disulfide interchange involves closure of 20-membered rings. Rate constants for reaction of GSH and CySH with the disulfide bonds of AVP and OT are 1–2 orders of magnitude larger than for reaction with disulfide bonds formed by two cysteine-containing peptides, which suggests that the disulfide bonds in the neurohypophyseal peptide hormones are strained. Equilibrium constants are also reported for reaction of GSH with the hexapeptide analogs of AVP and OT, pressinoic acid (PA), and tocinoic acid (TA). A reduction potential of −0.216 V was calculated for the disulfide bonds of OT and TA from the thiol/disulfide interchange equilibrium constants. Reduction potentials of −0.229 V and −0.227 V were calculated for the disulfide bonds in AVP and PA, respectively. The similarity of the reduction potentials for OT and TA and for AVP and PA indicates that the acyclic tripeptide tails of OT and AVP have little affect on the redox properties of their disulfide bonds.

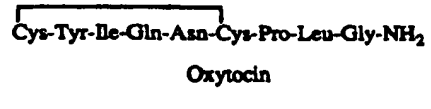
Thiol/disulfide interchange reactions provide a mechanism for the reversible formation of disulfide bonds in biological systems.[1] Such reactions are involved in maintaining the intracellular distribution of glutathione, coenzyme A, cysteine, and other thiols among their oxidized and reduced forms. The reversible formation of disulfide bonds is also utilized in biology as a cellular defense system, to regulate metabolism, and to transport reducing equivalents.[1b]

In previous studies, we have characterized the kinetics and equilibria of thiol/disulfide interchange reactions of biological monothiols.[2] These and other[3] studies have elucidated the molecular aspects of thiol/disulfide interchange reactions, including the effect of pH, thiol pK_A, and charge and steric factors. Mechanistically, thiol/disulfide interchange takes place via a simple nucleophilic displacement of a thiolate anion from the disulfide by another thiolate anion.[1b,2b,3a,d] No intermediates have been observed, which suggests a single transition state with a significant negative charge on the attacking and leaving thiolates and on the central sulfur atom.

In the next phase of this research program, we are studying the thiol/disulfide chemistry of peptides which have an intrapeptide disulfide bond. We report here the results of a study of the kinetics and equilibria of thiol/disulfide interchange reactions of the neurohypophyseal peptide hormones arginine vasopressin (AVP) and oxytocin (OT) with the tripeptide glutathione (γ-L-glutamyl-cysteinyl-glycine, GSH) and cysteine (CySH).



AVP



Oxytocin

AVP and OT have in common seven of their nine amino acids, a hexapeptide ring formed by residues 1–6 and an acyclic tripeptide tail. The hexapeptide ring in both AVP and OT is closed by a disulfide bond between cysteine residues at positions 1 and 6.

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The disulfide bond is one of the most important structural features of AVP and OT.[4] In early reports, their biological activity[5] was accounted for in terms of a mechanism which assumed that interaction with their receptors involved covalent bond formation via thiol/disulfide interchange reactions.[6] However, structure–activity studies with analogs have shown the disulfide bond is not involved in the mechanism of action, but rather it is the cyclic arrangement of amino acids 1–6 that is essential for high biological activity.[4] The cyclic arrangement need not involve a disulfide bond; for example, analogs of deaminooxytocin in which the disulfide group is replaced by CH_2S are biologically active. Thus, the main purpose of the disulfide bond in AVP and OT is to form and keep a molecular conformation suitable for noncovalent interaction with AVP and OT receptors.[7]

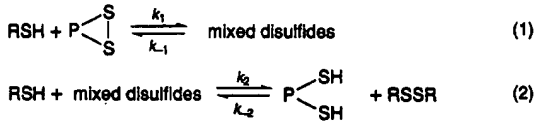
GSH and CySH were chosen for this study of the thiol/disulfide chemistry of AVP and OT because rate and equilibrium constants for their thiol/disulfide interchange reactions with a variety of other thiols and disulfides are available for comparison.[2a,3a] Also, GSH is the standard to which other thiols and disulfides are compared thermodynamically.[1b] Equilibrium constants and forward and reverse rate constants were determined for each step in the two-step reduction of AVP and OT by GSH and CySH, and redox potentials were calculated from the equilibrium constants for the overall reduction reactions.[8] Equilibrium constants were also determined for the reaction of GSH with the disulfide groups of the hexapeptide analogs of AVP and OT, pressinoic acid (PA), and tocinoic acid (TA). Major objectives of this study were to determine the tendency of the disulfide bonds in AVP and OT to undergo reduction by thiol/disulfide interchange, to characterize the kinetics of formation of the disulfide-containing 20-membered rings of AVP and OT by thiol/disulfide interchange, to determine redox potentials for the disulfide bonds in AVP and OT, and to determine the effect of the acyclic tripeptide tails on the thiol/disulfide interchange reactions and redox properties

Pressinoic Acid Tocinoic Acid

of AVP and OT. Such information is of interest not only with respect to the stability of the disulfide bonds of AVP and OT in biological systems, but also for understanding factors which influence the formation and stability of disulfide-containing loops in engineered peptides and proteins.

The Model

Reduction and formation of the disulfide bonds in AVP, OT, PA, and TA by thiol/disulfide interchange is summarized by the reaction scheme:

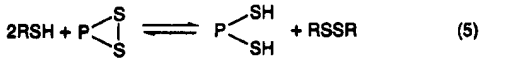


where RSH represents GSH and CySH. RSH reacts with the disulfide bond to form one of two possible mixed disulfides, which in turn react with RSH to give the reduced dithiol form of the peptide and RSSR.[9] Rate and equilibrium constants for reduction of the peptides by GSH and CySH and for formation of the peptide disulfide bonds by reaction of the reduced peptides with GSSG and CySSCy were determined by separation and analysis of reaction mixtures by high performance liquid chromatography (HPLC) using chromatographic conditions described previously.[10] To illustrate, the chromatogram for an AVP–GSH reaction mixture is shown in Figure 1. The kinetics and equilibria of the thiol/disulfide interchange reactions were characterized in terms of the total concentration of mixed disulfide, as summarized by eqs 1–4:

$$K_1 = \frac{[P\!<^{SSR}_{SH} + P\!<^{SH}_{SSR}]}{[RSH][P\!<^{S}_{S}]} \qquad (3)$$

$$K_2 = \frac{[P\!<^{SH}_{SH}][RSSR]}{[RSH][P\!<^{SSR}_{SH} + P\!<^{SH}_{SSR}]} \qquad (4)$$

The result of the overall reaction is reduction of the disulfide bond and formation of RSSR:



$$K_{ov} = \frac{[P\!<^{SH}_{SH}][RSSR]}{[RSH]^2[P\!<^{S}_{S}]} \qquad (6)$$

Thiol/disulfide interchange occurs by a simple S_N2 nucleophilic displacement, with the thiolate anion being the reactive form of RSH.[1b,2b,3a,d] Thus, the rates of thiol/disulfide interchange reactions are pH dependent, as are the equilibrium constants if the reactant and product thiols have different pK_A values. Conditional rate and equilibrium constants were measured at pH 7.00 and are expressed in terms of the total concentration of protonated and thiolate forms of the reactant and product thiols. Intrinsic rate and equilibrium constants were calculated in terms of the concentrations of the reactive

(4) Jošt, K. in *Handbook of Neurohypophyseal Peptide Hormones*; Jošt, K., Lebl, M., Brtnik, F., Eds.; CRC Press, Inc.: Boca Raton, FL, 1987; Vol. 1, part 2, 144–155.

(5) The major physiological activities of OT in mammals are milk ejection and uterine contraction; the major activities of AVP are vasoconstriction and antidiuretic functions.

(6) (a) Fong, C. T. O.; Schwartz, I. L.; Popenoe, E. A.; Silver, L.; Schoessler, M. A. *J. Am. Chem. Soc.* **1959**, *81*, 2592–2593. (b) Fong, C. T. O.; Silver, L.; Christman, D. R.; Schwartz, I. L. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1273–1277. (c) Rasmussen, H.; Schwartz, I. L.; Schoesler, M. A.; Hochster, G. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1278–1287. (d) Schwartz, I. L.; Fong, C.T.O.; Popenoe, E. A.; Silver, L.; Schoesler, M. A. *J. Clin. Invest.* **1959**, *38*, 1041. (e) Schwartz, I. L.; Rasmussen, H.; Schoesler, M. A.; Silver, L.; Fong, C.T.O. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1288–1298.

(7) Hormone–receptor thiol/disulfide interchange reactions take place; however, they apparently involve thiol groups on the receptor that are not concerned with hormonal activity.[4]

(8) Redox potentials determined for thiol/disulfide systems by direct electrochemical measurement are not reliable because typically the reactions are thermodynamically irreversible due to reaction with the electrode surface. Jocelyn, P. C. *Biochemistry of the Sulfhydryl Group*; Academic Press: New York, 1972.

(9) Formation of double mixed disulfides by reaction of the single mixed disulfides with RSSR and of dimers by reaction of single mixed disulfides with AVP or OT is also possible. Low peptide concentrations were used to minimize formation of dimers and there was no evidence for their formation in the experiments reported here.

(10) Yeo, P. L.; Rabenstein, D. L. *Anal. Chem.* **1993**, *65*, 3061–3066.

Table 1. Equilibrium Constants for Thiol/Disulfide Interchange Reactions[a]

	OT/GSH	AVP/GSH	OT/CySH	AVP/CySH	TA/GSH	PA/GSH
K_1, M^{-1}	140 ± 4	60 ± 2	67 ± 3	25.9 ± 0.3	130 ± 2	42 ± 1
K_2	0.26 ± 0.01	0.24 ± 0.03	0.15 ± 0.02	0.124 ± 0.007	0.28 ± 0.01	0.36 ± 0.01
K_{ov}, M^{-1}	36 ± 2	14.5 ± 1.8	10 ± 1	3.2 ± 0.2	35.8 ± 0.7	15.1 ± 0.5
f_1[b]	0.62 ± 0.01	0.69 ± 0.02	0.64 ± 0.02	0.65 ± 0.01	0.59 ± 0.01	0.73 ± 0.01
f_2[c]	0.38 ± 0.01	0.31 ± 0.02	0.36 ± 0.02	0.35 ± 0.01	0.41 ± 0.01	0.27 ± 0.01

[a] 25 °C and pH = 7.00; 0.15 M KCl. [b] Fractional concentration of the mixed disulfide which elutes first. [c] Fractional concentration of the mixed disulfide which elutes second.

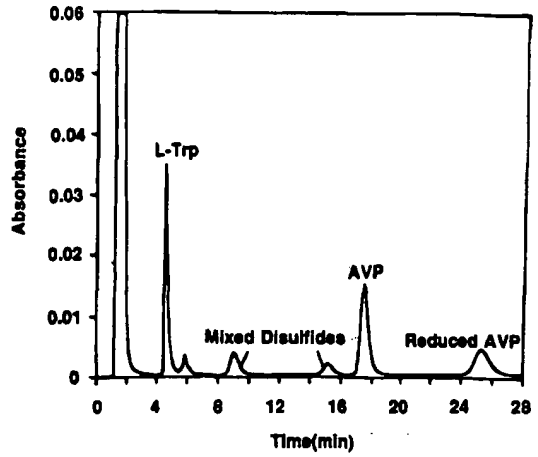


Figure 1. Chromatogram of an AVP/GSH reaction mixture at equilibrium. The initial concentrations were 32.6 μM AVP, 7.11 mM GSH and 1.23 mM GSSG at pH 7.0 and 25 °C. The peaks at 2 min. are for GSH, GSSG and the solvent front. The peak at ~6 min. is from the double mixed disulfide. The mobile phase contained 12% acetonitrile, 0.1 *M* NaH_2PO_4 and sufficient H_3PO_4 to adjust the pH to 2.5.

thiolate species from the conditional constants measured at pH 7.00.

Results

Thiol/Disulfide Interchange Equilibrium Constants. The equilibrium constants defined by eqs 1–6 were determined for reaction of GSH and CySH with AVP and OT and for reaction of GSH with PA and TA. Determination of equilibrium constants for the GSH/AVP system will be described to illustrate the procedures used. Peaks are observed for the native disulfide, reduced dithiol, and two mixed disulfide forms of AVP, and for the internal intensity standard L-tryptophan in the chromatogram for an equilibrium reaction mixture (Figure 1). The peaks for AVP and reduced AVP were assigned using authentic samples of the two compounds. Evidence for assignment of the two peaks at 9 and 15 min to single mixed disulfides is that they are observed only in the presence of GSH and constant values are obtained for K_1 and K_2 over a range of [GSH]/[GSSG] ratios.[11-18] K_1 depends on 1/[GSH], while K_2 depends on [GSSG]/[GSH]. GSH and GSSG are essentially unretained with the mobile phase used, and thus their peaks are in the peak for the solvent front. The reaction solution contained known, excess concentrations of GSH and GSSG, and thus it was only necessary to determine the concentrations of the various forms of AVP by HPLC analysis.[10,14] Concentrations of the disulfide and reduced forms of AVP were determined from the areas of their peaks and the internal intensity standard by using the appropriate calibration factors.[10] Since pure samples of the mixed disulfides were not available for calibration of the detector response, the total concentration of mixed disulfide was calculated to be the difference between the initial AVP concentration and the sum of the concentrations determined for AVP and reduced AVP.

To establish that equilibrium is reached, a procedure was used in which equilibrium was approached from both directions.[10] AVP was reacted with a GSH/GSSG redox buffer. Aliquots were removed as a function of time, quenched, and analyzed by HPLC until equilibrium was reached, as indicated by no further change in the concentrations of the disulfide, dithiol, and mixed disulfide forms of AVP. The equilibrium was then perturbed by addition of a known amount of GSSG, and aliquots were removed and analyzed as the system approached equilibrium from the opposite direction. Equilibrium constants were calculated from the concentrations determined for the disulfide, dithiol, and mixed disulfide forms of AVP and the known concentrations of GSH and GSSG, corrected to account for changes in concentration due to the reactions in eqs 1 and 2. To illustrate, the values obtained for K_1, K_2, and $K_{overall}$ from the first equilibrium in a typical experiment are 62.2 M^{-1}, 0.223, and 13.9 M^{-1}, respectively, while those obtained from the second equilibrium are 61.6 M^{-1}, 0.216, and 13.3 M^{-1}. The average of 12 values for K_1, K_2, and K_{ov} from six experiments of this type for the AVP/GSH system are reported in Table 1. Typically the AVP concentration was in the 50 μM range, while GSH and GSSG concentrations were in the 7–20 mM and 1–4 mM range, respectively.

Equilibrium constants determined by the above procedure are reported in Table 1 for all the systems studied.

Microscopic equilibrium constants in terms of the individual mixed disulfides (Figure 1) can be calculated from the values for K_1 and K_2 in Table 1 and the fractional concentrations reported in Table 1 for each of the mixed disulfides. For example, using the results reported in Table 1, microscopic equilibrium constants of K_{1m} = 41 M^{-1} and K_{2m} = 0.35 are calculated for the first AVP–GSH mixed disulfide to elute, where K_{1m} is for the reaction of AVP with GSH to form the mixed disulfide and K_{2m} is for reaction of GSH with the mixed disulfide. For the second mixed disulfide to elute, K_{1m} = 19 M^{-1} and K_{2m} = 0.77. The fractional concentrations of the individual mixed disulfides were determined from the ratios of the peak areas for the mixed disulfides.

Rate Constants for Thiol/Disulfide Interchange. Rate constants for the forward and reverse reactions in eqs 1 and 2 were determined for the OT/GSH, AVP/GSH, OT/CySH, and AVP/CySH systems. Rate constant k_1 was determined by measuring the concentration of OT or AVP

(11) Zhang, R.; Snyder, G. H. *J. Biol. Chem.* **1989**, *263*, 18472–18479.
(12) Huyghnes-Despointes, B. M. P.; Nelson, J. W. *Biochemistry* **1992**, *31*, 1476–1483.
(13) Zhang, R.; Snyder, G. H. *Biochemistry* **1988**, *27*, 3785–3794.
(14) Lin, T.-Y.; Kim, P. S. *Biochemistry* **1989**, *28*, 6282–5287.

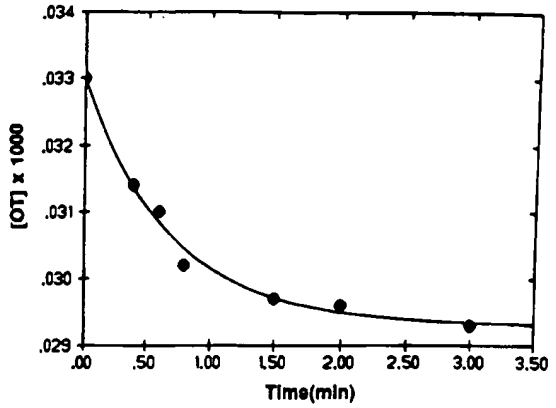


Figure 2. The concentration of OT as a function of time for the reaction of 33.0 μM OT with 0.849 mM GSH in 0.15 *M* KCl at pH 5.5 and 25 °C.

as a function of time after GSH or CySH was added to a solution of the peptide. The reactions were run under pseudo-first-order conditions by using an excess concentration of GSH or CySH. Typical time course data for the reaction of OT with GSH at pH 5.5 are presented in Figure 2. Peaks were observed for OT and the OT–GSH mixed disulfides in chromatograms for samples taken over the time period in Figure 2. However, no peaks were observed for the reduced, dithiol form of OT. Thus, the results in Figure 2 indicate that the reaction mixture rapidly comes to a pseudoequilibrium between OT and its mixed disulfides.[15] Pseudo-first-order rate constant k'_1 was obtained by fitting the time course data to the equation for a first order, reversible reaction:[16]

$$A_t = \frac{A_o(K'_1 + \exp(k'_1(1 + 1/K'_1)t))}{(K'_1 + 1)\exp(k'_1(1 + 1/K'_1)t)} \qquad (7)$$

where A_t is the concentration of OT at time t, A_o the initial concentration of OT, k'_1 the pseudo-first-order rate constant for reaction of OT with GSH, and K'_1 a pseudoequilibrium constant (K'_1 = [mixed disulfides]/[OT] = k'_1/k_{-1}). The second-order rate constant k_1 at pH 5.5 was then calculated from k'_1 using the relationship $k'_1 = k_1$[GSH]. Rate constant k_1 at pH 7.0 was calculated from the rate constant at pH 5.5 using the relationship $k_1(7.0) = k_1(5.5)(\alpha_{7.0}/\alpha_{5.5})$ where $\alpha_{7.0}$ and $\alpha_{5.5}$ ($\alpha = K_A/(K_A + [H^+])$) are the fractional concentrations of GSH in the thiolate form at pH 7.0 and 5.5.[17]

This treatment assumes that the reaction of GSH with OT to form the mixed disulfides is second order.[18] To verify that this is the case, time course data were measured for the following reactant concentrations: 18.4 μM OT and 0.971 mM GSH, 36.9 μM OT and 0.971 mM GSH, and 36.9 μM OT and 0.466 mM GSH, all at pH 5.5. Values of 3.66, 3.43, and 3.76 M^{-1} s^{-1} were obtained for k_1 from the time course data.

Values determined for rate constant k_1 by the above procedure for all the systems studied are listed in Table 2. Also listed are values calculated for k_{-1} using the relation $k_{-1} = k_1/K_1$.

Rate constant k_{-2} for reaction of the reduced dithiol form of the peptides with GSSG or CySSCy was determined by measuring the concentration of reduced peptide as a function of reaction time. The reactions were run at pH 7.00 under pseudo-first-order conditions with respect to reduced peptide. Rate constant k_{-2} was obtained from the initial slope of the time course data, and k_2 was then calculated using the relation $k_2 = K_2k_{-2}$. The results obtained for the OT/GSH, AVP/GSH, OT/CySH, and AVP/CySH systems are reported in Table 2.

Table 2. Rate Constants for Thiol/Disulfide Interchange Reactions[a]

	OT/GSH	AVP/GSH	OT/CySH	AVP/CySH
k_1, M^{-1} s^{-1}	110 ± 6	38 ± 6	41 ± 3	36 ± 6
k_{-1}, s^{-1}	0.76 ± 0.04	0.63 ± 0.10	0.61 ± 0.04	1.4 ± 0.2
k_2, M^{-1} s^{-1}	0.84 ± 0.13	0.74 ± 0.09	1.3 ± 0.2	1.2 ± 0.2
k_{-2}, M^{-1} s^{-1}	3.2 ± 0.5	3.1 ± 0.1	8.8 ± 0.1	10.0 ± 1.4

[a] 25 °C and pH = 7.00; 0.15 M KCl.

Table 3. Intrinsic Rate and Equilibrium Constants for Thiol/Disulfide Interchange[a]

	OT/GSH	AVP/GSH	OT/CySH	AVP/CySH
K^i_{OV}, M^{-1}	1 190	539	23	8.3
k^i_1, M^{-1} s^{-1}	10 400	3 600	1 020	900
k^i_{-1}, s^{-1}	9	6	7	13
k^i_2, M^{-1} s^{-1}	79	70	32	30
k^i_{-2}, M^{-1} s^{-1}	37	29	100	94

[a] 25 °C and 0.15 M KCl.

Discussion

The fraction of thiol in the reactive thiolate form, and thus rate and equilibrium constants for thiol/disulfide interchange reactions, are pH dependent. The equilibrium constants reported in Table 1 and the rate constants in Table 2 are conditional constants for pH 7.00. pH independent, intrinsic rate constants and overall equilibrium constants are reported in Table 3. The intrinsic equilibrium constants K^i_{OV} were calculated using the relation $K^i_{OV} = (\alpha_2/\alpha^2_{RSH})K_{OV}$ where α_2 is the fractional concentration of reduced peptide in the dithiolate form and α_{RSH} the fractional concentration of GSH or CySH in the thiolate form at pH 7.00. α_2 was calculated to be 3.7×10^{-3}, 4.2×10^{-3}, 6.2×10^{-5}, and 9.3×10^{-5} using literature pK_A values for the two thiol groups of reduced OT, AVP, TA, and PA, respectively.[19] Intrinsic rate constants were calculated from the conditional rate constants in Table 2 using relationships of the type $k_1 = \alpha_{RS}$-k^i_1, where k^i_1 is the intrinsic rate constant and α_{RS} the fractional concentration of RSH in the thiolate form. k^i_1 and k^i_2 were calculated using pK_A values of 8.97 and 8.38 for GSH and CySH, respectively.[17] k^i_{-2} and k^i_{-1} were estimated using an average of α values calculated for the two thiol groups of the reduced peptides.[19]

(15) Reduction of the intramolecular disulfide bond in other peptides also takes place in distinct kinetic phases, with the first step fast relative to the second step.[13]

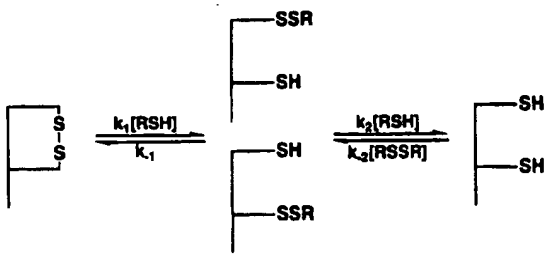
(16) Amdur, I.; Hammes, G. G. *Chemical Kinetics: Principles and Selected Topics*; McGraw Hill: New York, 1966. Note: eq 8 in ref 10 is incorrect; however, the correct equation was used to fit the time course data in ref 10.

(17) The rates of the reaction of GSH and CySH with AVP and OT are too fast at pH 7.00 and the concentrations used in this study to characterize by removal of aliquots followed by analysis by HPLC. The rates of the reactions with GSH and CySH are slower by factors of 0.032 and 0.033, respectively, at pH 5.5, because smaller fractions of GSH and CySH are present in the reactive thiolate forms. These factors were calculated using pK_A values of 8.97 and 8.38 for the thiol groups of GSH and CySH, respectively (Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797–2803 and Backs, S. J.; Rabenstein, D. L. *Inorg. Chem.* **1981**, *20*, 410–416).

(18) It is well established that thiol/disulfide interchange reactions are mechanistically simple second-order S_N2 displacement reactions.[2b,3a]

(19) Noszál, B.; Guo, W.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 2327–2334.

Kinetics and Equilibria at pH 7.00. Since the cyclic arrangement of amino acids 1–6 is essential for high biological activity,[4] rate and equilibrium constants of thiol/disulfide interchange reactions of OT and AVP under near physiological conditions are of interest. It is convenient to discuss the stepwise kinetics for reduction and formation of the peptide disulfide bonds by thiol/disulfide interchange using the reaction scheme:



where k_1[RSH], k_2[RSH], and k_{-2}[RSSR] are pseudo-first-order rate constants.

The results reported in Table 2 for the reaction of AVP and OT with GSH and CySH indicate that the second-order rate constant, k_1, for the first step in the overall reduction of the disulfide bond in AVP and OT by thiol/disulfide interchange is much larger than k_2, i.e. the second step in the overall reduction of peptide by RSH is rate determining. For example, k_1 for reduction of OT by GSH is 130 times k_2. For comparison, rate constants for the second-order reaction of GSH and CySH with GSSG at pH 7.00 are 0.41 and 0.33 M^{-1} s^{-1}, respectively,[2a,g], i.e. similar in magnitude to k_2. Using rate constants k_2 and k_{-2} for the OT/GSH and AVP/GSH systems as characteristic thiol/disulfide interchange rate constants (k_{ex}) for these peptides in the absence of ring strain, a value of 1.5 M^{-1} s^{-1} is obtained for k_{ex} after correcting for statistical factors. Rate constant k_1 for the OT/GSH and AVP/GSH systems is 73 and 25 times larger than k_{ex}. Thus, it is evident that the second step is rate determining not because it is slower than normal for thiol/disulfide interchange reactions, but rather because the first step is 1–2 orders of magnitude faster than normal. This suggests that the disulfide bonds in OT and AVP are somewhat strained.[20]

Even though $k_1 \gg k_2$, the peptides are not all rapidly converted to mixed disulfide by reaction with GSH or CySH. Rather, the initial reaction of peptide with RSH is rapidly reversed and a pseudoequilibrium is established between peptide and the peptide-RSH mixed disulfides, as shown by the kinetic data in Figure 2 for reaction of OT with GSH.[15] For example, taking the OT/GSH system as an example, $k_{-1} > k_1$[GSH], when [GSH] < 0.007 M. Thus, under the conditions used in this study, the mixed disulfides are relatively rapidly converted back to peptide by intramolecular thiol/disulfide interchange and a pseudoequilibrium is established. At the same time, some mixed disulfide reacts further with RSH. The relative rates of the competing intra- and intermolecular processes depend on the concentration of RSH. For example, at [GSH] < 0.9 M, $k_{-1} > k_2$[GSH] and intramolecular disulfide bond formation is faster than reaction with another molecule of GSH to form reduced OT and GSSG. This suggests that, in human blood plasma where the concentration of nonprotein thiol is $\ll$0.9 M,[21] mixed disulfides formed by reaction of AVP and OT with nonprotein thiols will undergo intramolecular thiol/disulfide interchange to reform biologically active AVP and OT rather than react with another molecule of nonprotein thiol to give the reduced dithiol forms of AVP and OT. At the plasma concentration of GSH,[21] the equilibrium concentration of mixed disulfides is also predicted to be small.[22]

The reaction of reduced OT and AVP with GSSG or CySSCy to form the native disulfide forms of the peptides is of interest since it involves closing a 20-membered hexapeptide ring by formation of an intrapeptide disulfide bond. The overall reaction involves two steps. In the first step, reduced peptide reacts with GSSG or CySSCy to form the mixed disulfide, which in turn reacts by intramolecular thiol/disulfide interchange to form the peptide disulfide bond. Which of the two steps is rate determining depends on whether k_{-1} or the pseudo-first-order rate constant k_{-2}[RSSR] is larger. Taking the reaction of reduced OT with GSSG as an example, the first step will be rate limiting when [GSSG] < 0.24 M, i.e. closure of the 20-membered ring by intramolecular thiol/disulfide interchange is fast relative to the reaction of reduced OT with GSSG.

The formation of peptide rings by intramolecular thiol/disulfide interchange has been analyzed previously for several peptides and proteins in terms of an effective concentration, C_{eff}.[12,14,20,23] C_{eff} represents a ratio of equilibrium constants for otherwise identical intra- and intermolecular processes. In such studies, glutathione is generally used as the reference thiol, and it can be shown that:

$$C_{eff} = \frac{K_{intra}}{K_{inter}} = \frac{|P\langle^{S}_{S}|[GSH]|^2}{|P\langle^{SH}_{SH}|[GSSG]}$$

In terms of the equilibrium constants in Table 1, $C_{eff} = 1/K_{OV}$. The magnitude of C_{eff} has been interpreted to indicate the tendency of the peptide or protein to keep the cysteine thiols in proximity even in the absence of a disulfide bond.[20] For example, C_{eff} for disulfide bond formation at the active site of thioredoxin at pH 8.7 is 10 M.[14] In the presence of urea, C_{eff} for thioredoxin decreases, reaching a minimum value of 0.026 M in 7.7 to 9 M urea. For comparison, C_{eff} for the random coil peptide [Ac-Cys-(Gly)$_6$-Cys-NH$_2$] is constant at ~0.060 M at pH 8.7 and urea concentrations ranging from 0 to 7 M.[14] The values calculated for C_{eff} from K_{OV} in Table 1 are 0.07 M for the AVP/GSH system and 0.03 M for the OT/GSH system. By comparison to the value of 0.060 M for the [Ac-Cys-(Gly)$_6$-Cys-NH$_2$]/GSH system[14] and the values reported for random polymers,[13,14] we can conclude that the two cysteines of reduced AVP and OT are not constrained to be closer together than would be the case for an unstructured peptide. It also is of interest that C_{eff} = 0.07 M for the PA/GSH system and 0.03 M for the TA/GSH system, i.e. the acyclic tripeptide tails of AVP and OT seem to have no effect on the proximity of the two thiol groups of reduced AVP and OT.

(20) Creighton, T. E. *Methods Enzymol.* **1986**, *131*, 83–106.

(21) The concentration of GSH in human plasma is in the range of 2 μm: Henning, S. M.; Zhang, J. Z.; McKee, R. W.; Swendseid, M. E.; Jacob, R. A. *J. Nutr.* **1991**, *121*, 1969–1975.

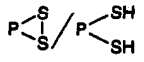
(22) Using the equilibrium constants in Table 1 and a plasma GSH concentration of 2 μM, the ratio of mixed disulfides to disulfide is predicted to be approximately 3×10^{-4} for AVP and 1×10^{-3} for OT at equilibrium.

(23) Creighton, T. E. *Biopolymers* **1983**, *22*, 49–58.

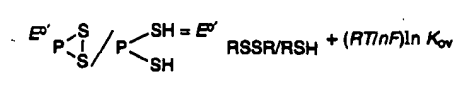
Intrinsic Rate and Equilibrium Constants. The intrinsic rate constants in Table 3 are all larger than the corresponding values in Tables 1 and 2 since the thiols are only fractionally deprotonated at pH 7.00. The differences in k_1^i and k_2^i are larger for the peptide/GSH systems than for the peptide/CySH systems because the pK_A is larger for GSH than for CySH. Thus, for the peptide/GSH systems, $k_1^i \gg k_1$ and $k_2^i \gg k_2$ and, as a consequence, $K_{ov}^i \gg K_{OV}$. The inherent nucleophilicity is less for CySH, and thus k_1^i, k_2^i, and K_{ov}^i are similar for the peptide/CySH systems. However, the values for the peptide/GSH and peptide/CySH systems are similar at pH 7 because the greater fraction of CySH in the reactive thiolate form compensates for its lower nucleophilicity.

The intrinsic rate and equilibrium constants in Table 3 can be compared with values reported for thiol/disulfide interchange reactions of other peptides which contain two cysteines.[11,13] For example, rate constants k_1 and k_{-1} and equilibrium constant K_1 were reported for reaction of 12 peptides of the type Cys-Xaa$_m$-Cys where m is the number of amino acids between the cysteines, with GSH in 3 M guanidine HCl at pH 6.9. Intrinsic constants were calculated from the pH 6.9 values by assuming the thiol pK_A values to all be 8.9.[11] Of particular interest with respect to the results in Table 3 are rate constants for the series Cys-Ala$_3$-Cys, Cys-Ala$_4$-Cys, and Cys-Ala$_5$-Cys. The values reported for k_1^i are 50, 16, and 18 M^{-1} s^{-1}, respectively, values reported for k_{-1}^i are 0.90, 0.90, and 0.55 s^{-1}, and values reported for K_1^i are 56, 18, and 33 M^{-1}. Rate constant k_{-1}^i is of particular interest for it involves peptide conformational transitions that bring a cysteine residue and a mixed disulfide bond into proximity for formation of an intramolecular disulfide bond.[20] Assuming that the k_{-1}^i value for Cys-Ala$_4$-Cys is a good reference value for a random coil peptide with two cysteines separated by four amino acids, the somewhat larger k_{-1}^i values in Table 3 suggest that for OT and AVP conformations in which the thiol and mixed disulfide groups are in close proximity are more populated than for completely random coil peptides. This conclusion must be considered somewhat tentative, however, since the values calculated for the intrinsic constants for the Cys-Ala$_m$-Cys series are dependent on the assumption that the pK_As for all the thiol groups are 8.9.[11] It also is of interest to note that k_1^i for OT and AVP is much larger than for Cys-Ala$_m$-Cys with m = 3, 4, and 5, which is further evidence that the disulfide bonds in OT and AVP are strained.

Redox Potentials of the Peptide Disulfide Bonds. A major objective of this research has been to quantitatively characterize the oxidation–reduction chemistry of the disulfide group in AVP and OT. Equilibrium constants for the overall reduction of the peptide disulfide bonds by GSH and CySH are related to half-cell potentials for the



and RSSR/RSH redox couples:



The redox potentials listed in Table 4 were calculated using the values for K_{OV} in Table 1 and reference values of $E^{o'}_{GSSG/GSH} = -0.262$ V and $E^{o'}_{CySSCy/CySH} = -0.245$ V.[24]

Table 4. Redox Potentials for Oxytocin, Arginine Vasopressin, Tocinoic Acid, and Pressinoic Acid[a,b]

peptide	thiol/disulfide system	$E^{o'}$ (V)
OT	OT/GSH	−0.216
OT	OT/CySH	−0.216
TA	TA/GSH	−0.216
AVP	AVP/GSH	−0.228
AVP	AVP/CySH	−0.230
PA	PA/GSH	−0.227

[a] pH 7.0 and 25 °C. 0.15 M KCl. [b] The uncertainty estimated for each $E^{o'}$ value on the basis of the uncertainty in the K_{OV} values is ±0.001 − 0.002 V; however, the actual uncertainty is probably larger due to uncertainty in $E^{o'}_{GSSG/GSH}$ and $E^{o'}_{CySSCy/CySH}$.[24]

There is excellent agreement between the two redox potentials calculated for OT from K_{OV} for the OT/GSH and OT/CySH systems. The same is true for the redox potentials for AVP. This is significant since the redox potentials serve as a check on the validity of the equilibrium constants. It also is of interest to note that $E^{o'}$ is essentially identical for OT and TA and for AVP and PA, which suggests that the acyclic tripeptide tails of OT and AVP apparently have no affect on the redox properties of their disulfide bonds.

It is of interest to compare the results in Table 4 to $E^{o'}$ values for other intramolecular disulfide bonds. $E^{o'}$ values reported recently for molecules which form 5–11-membered disulfide-containing rings range from −0.354 to −0.240 V.[25] For example, the $E^{o'}$ values for dithiothreitol (DTT), lipoic acid, and 6,6′-sucrosedithiol, which form 6-, 5- and 11-membered rings, are −0.327, −0.288, and −0.245 V, respectively. The $E^{o'}$ values for OT and AVP are less reducing; however, they are larger than might be expected, considering that formation of their disulfide bonds involves closure of 20-membered rings.

Experimental Section

Chemicals. AVP, OT, PA, and TA were obtained from Bachem Inc., Torrance, CA. HPLC traces provided by the supplier showed that >98% of the peptide present was AVP, OT, PA, or TA. Peptide content was determined by 500-MHz ^{1}H NMR to be in the range 70–90%, the remainder being water, in good agreement with the certificate of analysis provided by the supplier. Dithiothreitol (DTT) was obtained from Aldrich Chemical Co. Oxidized DTT, GSH, the sodium salt of GSSG, the hydrochloride forms of CySH and CySSCy, and *N*-acetyl-L-leucine (Ac-Leu) and *N*-acetyl-L-phenylalanine (Ac-Phe) were obtained from Sigma Chemical Co. Mobile phases used in the HPLC experiments and buffer solutions were prepared using sodium dihydrogen phosphate, sodium acetate, potassium chloride, phosphoric acid (85%), and acetonitrile (Optima) from Fisher Scientific Co.

HPLC Apparatus. Reverse-phase HPLC separations were performed with a liquid chromatograph equipped with a 20 μL sample loop, a 100 × 3.2 mm ODS (C_{18}) column (particle size 3 μm, Bioanalytical Systems MF-6213), and a dual channel UV detector. The detector was set at 215 nm. Mobile phases were prepared by addition of NaH_2PO_4 (0.1 M final concentration) and acetonitrile to water which had been purified with a Millipore water purification system, and then the pH was adjusted to 2.5 with 85% H_3PO_4. On the basis of results of a previous study of the chromatography of the native disulfide and reduced dithiol forms of AVP, OT, PA, and TA,[10] a mobile phase containing 12% acetonitrile was used for the AVP/GSH, AVP/CySH, and TA/GSH systems, 14% acetonitrile for the PA/

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GSH system, and 18% acetonitrile for the OT/GSH and OT/CySH systems. Mobile phases were filtered through a 0.45 μm cellulose nitrate filter membrane (Whatman 7184 004) and sparged with helium gas for at least 15 min before use.

Concentrations were obtained from chromatographic peak areas by using L-tryptophan, Ac-Leu, or Ac-Phe as an internal intensity standard.[10] Calibration solutions of reduced AVP, OT, PA, and TA were prepared by reduction of stock solutions of the peptides with an excess of DTT.

Kinetic and Equilibrium Studies. Stock solutions of AVP, OT, PA, and TA were prepared by weighing 1–2 mg of peptide on a Mettler M5SA microbalance, transferring the peptide to a 5 or 10 mL volumetric flask, and then adding 0.05 M sodium acetate–0.05 M NaH_2PO_4 solution (pH 5.6) which had been deoxygenated by bubbling with either argon or nitrogen. In the procedure used to study the kinetics of the reaction of GSH and CySH with AVP and OT, aliquots of peptide and internal standard stock solutions were combined and the pH adjusted to 5.5.[17] The solution was deoxygenated and placed in a water bath (25 °C) in a nitrogen-filled glove bag. Reaction was initiated by addition of GSH or CySH stock solution (pH 5.5). Aliquots of the reaction mixture were removed at time intervals of 12 s or more and quenched by lowering the pH to ~3 with HCl. The quenched solutions were then analyzed by HPLC. The quenching procedure was verified previously by analyzing a quenched reaction mixture as a function of time.[10]

Rate constants were determined for the reaction of reduced AVP and OT with GSSG and CySSCy at pH 7.00 by a similar procedure. Reduced AVP and OT were prepared by electrochemical reduction at a mercury pool electrode for ~3 h.[26]

Equilibrium constants for the thiol/disulfide exchange reactions were determined at pH 7.00 by measuring the concentrations of the native disulfide, reduced dithiol and mixed disulfide forms of the peptides in solutions containing known, excess concentrations of CySH and CySSCy or GSH and GSSG, i.e. in solutions containing an RSH/RSSR redox buffer.[27] As described above, a procedure was used in which equilibrium was approached from both directions to ensure that equilibrium was achieved.

Acknowledgment. This research was supported in part by National Institutes of Health Grant GM 37000. A Dissertation Fellowship from UC Riverside (P.L.Y.) is gratefully acknowledged.

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Kinetics and Equilibria of the Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione

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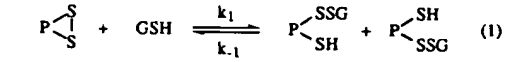
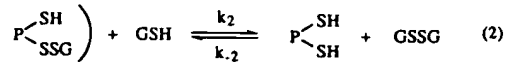
Rate and equilibrium constants are reported for the thiol/disulfide exchange reactions of the peptide hormone somatostatin with glutathione (GSH). GSH reacts with the disulfide bond of somatostatin to form somatostatin-glutathione mixed disulfides (Cys^3-SH, Cys^{14}-SSG and Cys^3-SSG, Cys^{14}-SH), each of which can react with another molecule of GSH to give the reduced dithiol form of somatostatin and GSSG. The mixed disulfides also can undergo intramolecular thiol/disulfide exchange reactions to re-form the disulfide bond of somatostatin or to interconvert to the other mixed disulfide. Analysis of the forward and reverse rate constants indicates that, at physiological concentrations of GSH, the intramolecular thiol/disulfide exchange reactions that re-form the disulfide bond of somatostatin are much faster than reaction of the mixed disulfides with another molecule of GSH, even though the intramolecular reaction involves closure of a 38-membered ring. Thus, even though the disulfide bond of somatostatin is readily cleaved by thiol/disulfide exchange, it is rapidly reformed by intramolecular thiol/disulfide exchange reactions of the somatostatin–glutathione mixed disulfides. By comparison with rate constants reported for analogous reactions of model peptides measured under random coil conditions, it is concluded that disulfide bond formation by intramolecular thiol/disulfide exchange in the somatostatin–glutathione mixed disulfides is not completely random, but rather it is directed to some extent by conformational properties of the mixed disulfides that place the thiol and mixed disulfide groups in close proximity. A reduction potential of −0.221 V was calculated for the disulfide bond of somatostatin from the thiol/disulfide exchange equilibrium constant.

Introduction

Disulfide bonds are structural elements in many biologically active peptides, including some peptide hormones and peptide toxins. For example, the nonapeptide hormones oxytocin (OT) and arginine vasopressin (AVP) both have a disulfide bond between cysteine residues at positions one and six, and the tetradecapeptide hormone somatostatin (SS) has a disulfide bond between cysteine residues at positions 3 and 14. In early reports, the biological activity of OT and AVP was accounted for in terms of a mechanism in which they interacted with their receptors by covalent bond formation via thiol/disulfide exchange reactions.[1] However, structure–activity studies have shown that the disulfide bond is not involved in the mechanism of action, but rather it is the cyclic arrangement of amino acids 1–6 that is essential for high biological activity.[2] The disulfide bonds in OT, AVP, and somatostatin serve to form and keep molecular conformations suitable for noncovalent interaction with their receptors.

Disulfide bonds are kinetically unstable structural elements, being readily cleaved by thiols via thiol/disulfide exchange.[3–7] In a previous study, we characterized the kinetics and equilibria for reduction and formation of the disulfide bonds of OT and AVP by thiol/disulfide exchange with glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) and oxidized glutathione (GSSG).[7,8] The reactions take place in two steps:





Rate constant k_1 was found to be 1–2 orders of magnitude larger than rate constants for thiol/disulfide exchange reactions of disulfide bonds between acyclic peptides. However, it was also found that, at physiological concentrations of GSH,[9] the intermediate mixed disulfides formed in this first step of the two-step reaction sequence undergo intramolecular thiol/disulfide exchange to re-form the native disulfide bonds much faster than they react with another molecule of GSH via the second reaction, even though the intramolecular thiol/disulfide exchange reactions involve closure of 20-membered rings. Furthermore, the rate constants for intramolecular thiol/disulfide exchange are significantly larger than reported for analogous reactions of model peptides under random coil conditions, which suggests that the AVP–GSH and OT–GSH mixed disulfides exist, at least part of the time, in precyclic conformations that place the thiol and mixed disulfide groups in close proximity.[7]

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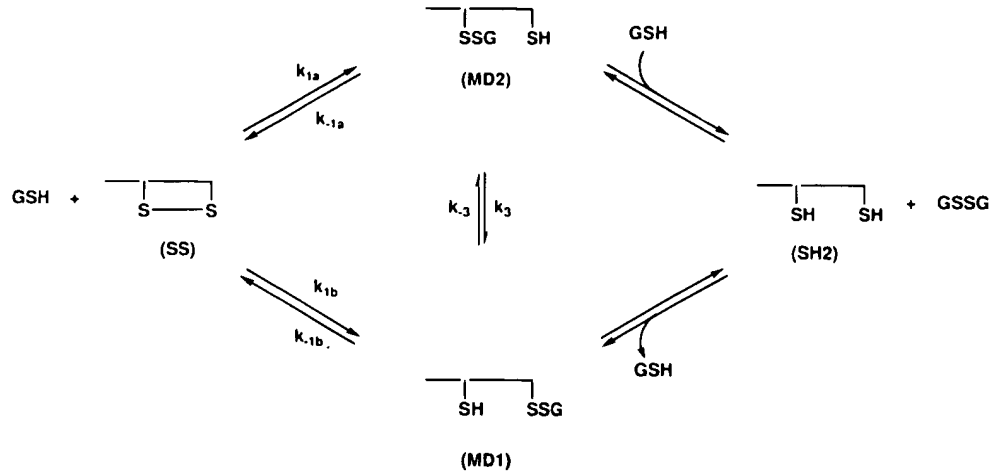

Figure 1. Thiol/disulfide exchange reactions of somatostatin (SS) with glutathione (GSH) and glutathione disulfide (GSSG). MD1 and MD2 are single somatostatin–glutathione mixed disulfides, with the disulfide bonds at Cys^{14} and Cys^{3} of somatostatin, respectively, and SH2 is the reduced, dithiol form of somatostatin.

In view of this finding, it is of interest to determine if the structurally important disulfide bonds in other peptide hormones are also rapidly reformed from their mixed disulfides by intramolecular thiol/disulfide exchange. In this paper, we report the results of a study of the kinetics and equilibria of the formation and reduction of the disulfide bond in somatostatin, which has an even larger (38 membered) disulfide-containing ring.

Ala-Gly-
Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Equilibrium constants and forward and reverse rate constants for the formation and reduction of the disulfide bond of somatostatin by thiol/disulfide exchange with the GSH/GSSG system were determined, including specific rate constants for the intramolecular thiol/disulfide exchange reactions of each of the two possible mixed disulfides. Also, the redox potential of the disulfide bond of somatostatin was determined from the equilibrium constant for its thiol/disulfide exchange reaction with GSH.

Results

The thiol/disulfide exchange reactions of somatostatin (SS) with GSH and GSSG are summarized in Figure 1. GSH reacts with the disulfide bond of SS to form a mixed disulfide (MD1 or MD2), which in turn reacts with another molecule of GSH to form reduced somatostatin (SH2) and GSSG. The reactions are reversible, and GSSG reacts with the thiol groups of SH2 to form MD1 or MD2, which in turn undergo intramolecular thiol/disulfide exchange to form the disulfide bond of SS. In addition, the mixed disulfides can interconvert by intramolecular thiol/disulfide exchange and they can react with another molecule of GSSG to form the double mixed disulfide (DMD) (not shown in Figure 1). Equilibrium constants were determined for each of the steps shown in Figure 1 and for formation of the double mixed disulfide. The kinetics of the reaction of SS with GSH to form MD1 and MD2 and of the intramolecular thiol/disulfide exchange reactions of MD1 and MD2 were characterized in terms of the rate constants shown in Figure 1, while the kinetics of the reaction of the mixed disulfides with another molecule of GSH and of SH2 with GSSG to form the mixed disulfides were characterized in terms of the rate constants defined by eq 2.

Thiol/Disulfide Exchange Equilibrium Constants. The equilibrium constants defined by eqs 3–11 were determined at 25 °C in pH 7.0 sodium phosphate buffer (0.075 M).

$$K_{1a} = \frac{[MD2]}{[SS][GSH]} \quad (3)$$

$$K_{1b} = \frac{[MD1]}{[SS][GSH]} \quad (4)$$

$$K_{2a} = \frac{[SH2][GSSG]}{[MD2][GSH]} \quad (5)$$

$$K_{2b} = \frac{[SH2][GSSG]}{[MD1][GSH]} \quad (6)$$

$$K_{1} = \frac{[MD1] + [MD2]}{[SS][GSH]} \quad (7)$$

$$K_{2} = \frac{[SH2][GSSG]}{([MD1]+[MD2])[GSH]} \quad (8)$$

$$K_{ov} = \frac{[SH2][GSSG]}{[SS][GSH]^2} \quad (9)$$

$$K_{3} = \frac{[MD1]}{[MD2]} \quad (10)$$

$$K_{4} = \frac{[DMD][GSH]^2}{[SH2][GSSG]^2} \quad (11)$$

The concentrations of SS and SH2 and of the two somatostatin–glutathione mixed disulfides were determined in equilibrium mixtures by HPLC. A typical chromatogram is shown in Figure 2. The peaks for SS and SH2 were assigned by comparison with chromatograms of each compound. The peaks labeled MD1 and MD2 were assigned previously by ^1H NMR to the specific

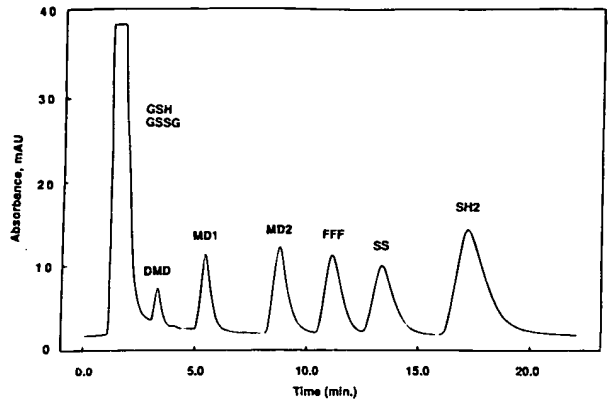

Figure 2. Chromatogram of a somatostatin/GSH reaction mixture at equilibrium (25 °C, pH 7.0 phosphate buffer). The peak labels are defined in the text. The concentrations determined from the peak areas are 5.73 μM SS, 14.9 μM SH2, 3.18 μM MD1, and 5.73 μM MD2. The concentration of the internal standard FFF was 15.0 μM. The mobile phase contained pH 3.0 phosphate buffer (0.1 M) and 26% acetonitrile.

somatostatin–glutathione mixed disulfides[10]

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
|
γ–Glu–Cys–Gly

(MD1)

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
|
γ–Glu–Cys–Gly

(MD2)

and the peak labeled DMD is for the double mixed disulfide

Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
| |
γ–Glu–Cys–Gly γ–Glu–Cys–Gly

(DMD)

The peak labeled FFF is for (phenylalanyl)(phenylalanyl)-phenylalanine, which was added as an internal intensity standard.

To establish that the reactions are reversible, equilibrium was approached from both directions.[11] After a solution prepared by combining stock solutions of GSH, GSSG, and somatostatin had reached equilibrium, as indicated by HPLC analysis, the equilibrium was shifted twice, first by addition of additional GSH and then by addition of additional GSSG. After each addition, aliquots of the resulting solutions were analyzed as a function of time until there was no further change in concentration. Equilibrium constants calculated using concentrations from the three separate equilibrium conditions were equal within experimental error. For example, the three values determined for K_1 from such an experiment were 121, 128, and 129 M^{-1} and the values determined for K_2 were 20.6, 20.8, and 22.4 M^{-1}. These results verify that the thiol/disulfide exchange reactions are reversible under the conditions used and that equilibrium was reached. The average values obtained for the equilibrium constants from 20 separate experiments over a range of reactant concentrations are reported in Table 1. Also listed for comparison in Table 1 are literature values for the analogous equilibrium constants for the OT/GSH and AVP/GSH systems.[7]

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Table 1. Equilibrium Constants for Thiol/Disulfide Exchange Reactions of Somatostatin, Arginine Vasopressin, and Oxytocin with Glutathione

	somatostatin[a–c]	arginine vasopressin[d]	oxytocin[d]
K_{1a}, M^{-1}	71 ± 9		
K_{1b}, M^{-1}	50 ± 6		
K_1, M^{-1}	121 ± 13	60	140
K_{2a}	0.35 ± 0.03		
K_{2b}	0.49 ± 0.06		
K_2	0.20 ± 0.02	0.24	0.26
K_{ov}, M^{-1}	24.7 ± 3.5	14.5	36
K_3	0.71 ± 0.06		
K_4	7 ± 2		
$E^{o\prime}$, V	−0.221	−0.228	−0.216

[a] 25 °C and 0.075 M pH 7.00 phosphate buffer. [b] Reactant concentrations covered the following ranges: 8–30 μM for the total somatostatin concentration; 4–18 mM for the GSH concentration; and 0.4–2 mM for the GSSG concentration. [c] The average relative standard deviation of the equilibrium constants is 13%. [d] 25 °C and pH = 7.00; 0.15 M KCl.[7]

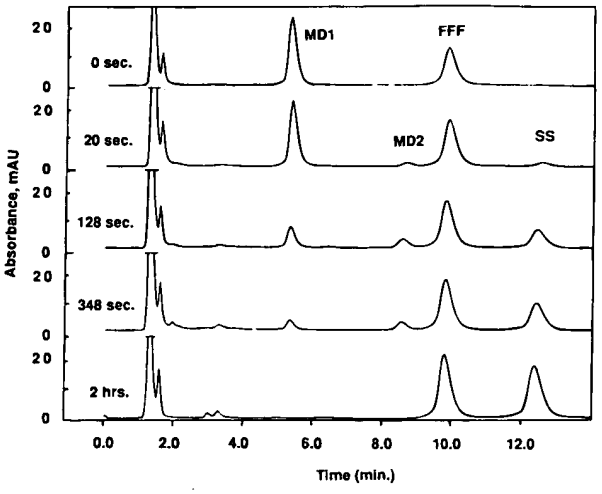

Figure 3. Chromatograms of samples taken as a function of time after raising the pH of an ~4 μM solution of MD1 and internal standard FFF to 7.0.

Kinetics of Thiol/Disulfide Exchange Reactions. Rate constants k_{-1a} and k_3 and rate constants k_{-1b} and k_{-3} (Figure 1) were determined directly by monitoring the conversion of MD2 and MD1, respectively, to SS and the other mixed disulfide. The procedure involved increasing the pH of solutions of MD1 or MD2 (at concentrations in the 1–10 μM range) from pH ~2.5, where thiol/disulfide exchange is very slow, to pH 7.0. Aliquots were then removed as a function of time, quenched and analyzed by HPLC. Representative chromatograms from a study of the conversion of MD1 to MD2 and SS are presented in Figure 3. At time 0, the chromatogram only shows peaks for MD1 and the internal intensity standard FFF. In the chromatogram for the aliquot taken at 20 s, peaks are also observed for both MD2 and SS, indicating that the cyclization reaction to form SS and the isomerization reaction to form MD2 are taking place at similar rates. As the reaction proceeds, the SS peak increases in intensity, and eventually both MD1 and the MD2 formed from MD1 are converted to SS. Since the solution only contains MD1 at $t = 0$, the cyclization and

Table 2. Rate Constants for Thiol/Disulfide Exchange Reactions of Somatostatin, Arginine Vasopressin, and Oxytocin with Glutathione[a]

	somatostatin[a]	arginine vasopressin[b]	oxytocin[b]
k_{1a}, M^{-1} s^{-1}	0.39 ± 0.09		
k_{-1a}, s^{-1}	0.0055 ± 0.0011		
k_{1b}, M^{-1} s^{-1}	0.49 ± 0.18		
k_{-1b}, s^{-1}	0.0097 ± 0.0034		
k_1, s^{-1}	0.88 ± 0.20	38	110
k_{-1}, s^{-1}	0.0072 ± 0.0020	0.63	0.76
k_3, s^{-1}	0.0030 ± 0.0006		
k_{-3}, s^{-1}	0.0039 ± 0.0009		
k_2, M^{-1} s^{-1}	1.83 ± 0.22	0.74	0.84
k_{-2}, M^{-1} s^{-1}	9.5 ± 0.6	3.1	3.2

[a] 25 °C, pH 7.00, 0.15 M phosphate buffer. [b] 25 °C, pH 7.00, 0.15 M KCl.[7]

Table 3. Intrinsic Equilibrium and Rate Constants for Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione

equilibrium constants		rate constants	
K^i_{1a}, M^{-1}	7.3	k^i_{1a}, M^{-1} s^{-1}	33.6
K^i_{1b}, M^{-1}	290	k^i_{-1a}, s^{-1}	4.58
K^i_1, M^{-1}	297	k^i_{1b}, M^{-1} s^{-1}	42.2
K^i_{2a}	0.036	k^i_{-1b}, s^{-1}	0.147
K^i_{2b}	2.8	k^i_3, s^{-1}	2.50
K^i_2	0.0355	k^i_{-3}, s^{-1}	0.059
K^i_{ov}, M^{-1}	10.5		
K^i_3	42.4		

interconversion reactions are the only reactions that are taking place initially, and thus, k_{-1b} and k_{-3} were obtained directly from the initial rates of formation of SS and MD2, respectively. Values determined for k_{-1a}, k_3 and k_{-1b}, k_{-3} from multiple experiments with MD2 and MD1, respectively, are presented in Table 2. Also listed in Table 2 are values for k_{1a} and k_{1b}, which were calculated from k_{-1a} and K_{1a} and k_{-1b} and K_{1b} using the relations $k_{1a} = K_{1a}k_{-1a}$ and $k_{1b} = K_{1b}k_{-1b}$.

The value listed in Table 2 for rate constant k_1 (eq 1) was calculated from k_{1a} and k_{1b} using the relation $k_1 = k_{1a} + k_{1b}$. Rate constant k_1 was also estimated from the initial rate of disappearance of SS by reaction with GSH. The procedure involved addition of GSH to a solution of SS and FFF, and then aliquots were removed as a function of time, quenched, and analyzed by HPLC. SS concentrations in the range 16–34 μM were used. To approximate pseudo-first-order reaction conditions, GSH concentrations in the 2–10 mM range were used. Because the rate of the reaction was found to be too fast at pH 7.0 to follow accurately by removing and quenching aliquots as a function of time, the reaction was run at lower pH where a smaller fraction of the GSH is in the reactive thiolate form. Rate constant k_1 was determined from the initial rate measured in 12 experiments in the pH range 5.3–6.2. Each of these rate constants was then used to estimate a value for k_1 at pH 7.00 by accounting for the increased concentration of the reactive thiolate form at pH 7.00; a pK of 8.93 was used for the thiol group of GSH.[12] An average value of 2.00 ± 0.55 M^{-1} s^{-1} was estimated for k_1 at pH 7.00.

Rate constant k_{-2} (eq 2) was determined by reaction of reduced somatostatin with GSSG at pH 7.0. The reduced somatostatin was prepared by electrochemical reduction of a SS/FFF solution.[13] The procedure for determination of k_{-2} involved addition of GSSG to the resulting solution of SH2/FFF at pH 7.0, and then aliquots were removed as a function of time, quenched, and analyzed by HPLC. Rate constant k_{-2} was determined from the initial rate of the reaction. The value reported in Table 2 is the average from six experiments in which the concentration of SH2 and GSSG were in the range of 20–50 μM and 0.3–0.5 mM, respectively. The value listed for rate constant k_2 was calculated from k_{-2} and K_2.

Discussion

Thiol/disulfide exchange occurs by an S_N2 displacement mechanism in which a thiolate anion, the reactive form of the thiol in thiol/disulfide exchange reactions, approaches the disulfide bond along its S–S axis.[3,5,14,15] The equilibrium and rate constants reported in Tables 1 and 2 are for pH 7.00, where only a fraction of the thiol groups of GSH, MD1, MD2, and the dithiol form of somatostatin are in the thiolate form, and thus they are conditional constants for pH 7.00. pH-independent, intrinsic equilibrium, and rate constants for the reactions in terms of thiolate species are reported in Table 3. The intrinsic equilibrium constants, K^i, were calculated using the values reported in Table 1 by accounting for the fractional thiolate concentrations at pH 7.00 with equations of the type $K^i_{1a} = (\alpha_{MD2}/\alpha_{GSH})K_{1a}$ where α is the fraction of the thiol group of the compound indicated in the thiolate form at pH 7.00. α was calculated to be 0.0116 for GSH, 0.0661 for the Cys^3 thiol group of MD1, 0.001 20 for the Cys^{14} thiol group of MD2 using pK_a values of 8.93 for GSH, 8.15 for the Cys^3 thiol group of MD1, and 9.92 for the Cys^{14} thiol group of MD2.[12,16,17] The fraction of the dithiol form of somatostatin with both thiol groups ionized was calculated to be 7.9×10^{-5} using pK_a values of 8.15 and 9.92 for the Cys^3 and Cys^{14} thiol groups. The intrinsic rate constants were calculated from the conditional rate constants in Table 2 using relationships of the type $k^i_{1a} = k_{1a}/\alpha_{MD2}$.

Kinetic Stability of the Disulfide Bond of Somatostatin. A main objective of this research was to characterize the kinetic stability of the disulfide bond of somatostatin with respect to cleavage by thiol/disulfide exchange. The results in Table 2 indicate that the disulfide bond of somatostatin is readily cleaved by a two-step reaction with GSH and that the rate constants for its two possible reactions with GSH in the first step (k_{1a} and k_{1b} in Figure 1) are essentially equal. Also, they are of a magnitude that is typical of rate constants for reaction of thiol-containing amino acids and peptides with intermolecular peptide disulfide bonds, which suggests that, even though it is part of a ring system, the disulfide bond of somatostatin is not particularly unstable and it reacts as a normal disulfide bond. For comparison, rate constants for the second-order reaction of GSH and cysteine with the disulfide bond of GSSG at pH 7.0 are 0.41 and 0.33 M^{-1} s^{-1}, respectively.[6] This is in contrast

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(16) The pK_a's of the mixed disulfides of somatostatin cannot be measured due to intramolecular thiol/disulfide exchange. The pK_a of Cys^3 was estimated to be 8.15 using the pK_a value reported for Cys^6 of arginine vasopressin in D_2O.[17] The pK_a of Cys^{14} was estimated to be 9.92 using the pK_a's reported for Cys^6 of tocinoic acid in D_2O.[17] The pK_a values for D_2O solution were converted to H_2O solution using the relation $pK_a(H_2O) = pK_a(D_2O) - 0.50$.[6]
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to the rate constants measured for reaction of GSH with the disulfide bonds of arginine vasopressin and oxytocin (k_1, eq 1), which are significantly larger than rate constants for typical thiol/disulfide exchange reactions,[7] and they are larger than rate constant k_1 for somatostatin.[18]

With respect to the kinetic stability of somatostatin, it is important to consider the fate of the mixed disulfides formed in the first step. The mixed disulfides can react with another molecule of GSH to give fully reduced somatostatin in the second step or they can undergo intramolecular thiol/disulfide exchange to re-form the disulfide bond of somatostatin. Rate constants k_{-1} and k_2 (eqs 1 and 2) can be used to determine the relative tendencies for reaction by these two pathways if k_2 is converted to the pseudo-first-order rate constant k_2' (= k_2[GSH]). Using the results in Table 2 for somatostatin, it can be shown that $k_{-1} > k_2'$ when [GSH] < 0.004 M; i.e., intramolecular thiol/disulfide exchange to re-form the disulfide bond of somatostatin is faster than reaction with another molecule of GSH when [GSH] < 0.004 M. This suggests that in human blood plasma, where the concentration of GSH and other nonprotein thiols is much less than 0.004 M,[9,19] mixed disulfides of somatostatin will tend to undergo intramolecular thiol/disulfide exchange to re-form the native disulfide bond rather than react with another molecule of thiol to give the reduced, dithiol form of somatostatin. That is, even though the disulfide bond of somatostatin is susceptible to cleavage by thiol/disulfide exchange, biologically active somatostatin is readily re-formed by intramolecular thiol/disulfide exchange. This is an important concept to consider when investigating the behavior of other disulfide-containing peptide hormones in biological systems and the use of disulfide bonds as structural elements in peptide and peptidomimetic drugs.

A similar analysis using the rate constants in Table 2 for arginine vasopressin and oxytocin indicates that the tendency for formation of their disulfide bonds by intramolecular thiol/disulfide exchange is even greater.[7] Specifically, $k_{-1} > k_2'$ when [GSH] < 0.85 and 0.90 M for arginine vasopressin and oxytocin, respectively. This even greater tendency for intramolecular thiol/disulfide exchange is most likely because smaller rings are formed by the disulfide bond in arginine vasopressin and oxytocin (20-membered as compared to the 38-membered ring of somatostatin). Nevertheless, the rate constant k_{-1} for somatostatin is surprisingly large. This can be seen, for example, by comparison of k_{-1} to rate constants reported for the analogous reactions of a homologous series of peptides of the type Cys-(Ala)$_n$-Cys, where n varies from 1 to 5, which corresponds to ring sizes of 11, 14, 17, 20, and 23 atoms for the disulfide form of the peptides.[20] The rate constants for the homologous series were measured under conditions where the mixed disulfides are random coil, and they were reported as intrinsic rate constants, k'_{-1}, i.e., rate constants for conditions where the reacting thiol groups are completely in the deprotonated thiolate form. For purposes of comparison with the values reported in Table 2, we have converted them to conditional rate constants at pH 7.0 by accounting for the protonation state of the thiol group at pH 7.0 using the relation $k_{-1} = \alpha k'_{-1}$, where α is the fraction in the thiolate form.[21] The values calculated for k_{-1} are 0.06, 0.12, 0.011, 0.011, and 0.0068 s^{-1} for $n = 1$, 2, 3, 4, and 5, respectively. Comparison with the values listed in Table 2 indicates that k_{-1} for arginine vasopressin and oxytocin is some 60–70 times larger than k_{-1} for the $n = 4$ random coil homolog, which also forms a 20-membered ring. No rate constants are available for homologs that form a 38-membered ring for comparison with k_{-1} for somatostatin. However, k_{-1} for somatostatin is essentially the same as k_{-1} for the $n = 5$ homolog, which forms a 23-membered ring; considering the steady decrease in k_{-1} for the series of random coil homologs as the size of the ring increases, it seems likely that k_{-1} for reaction of a random coil mixed disulfide to form a 38-membered ring would be significantly less than is found for somatostatin. That is, this analysis suggests that disulfide bond formation by intramolecular thiol/disulfide exchange in the somatostatin–glutathione mixed disulfides is not completely random, but rather it is directed to some extent by conformational properties of the mixed disulfides that place the thiol and the mixed disulfide groups in close proximity.

That some fraction of the somatostatin–glutathione mixed disulfides exist in precyclic conformations is supported by evidence from ^{1}H NMR studies of the two somatostatin–glutathione mixed disulfides.[10] ^{1}H NMR results, as well as results from semiempirical energy calculations and time-resolved fluorescence spectroscopy experiments, indicate that the cyclic disulfide form of somatostatin exists as an equilibrium of several rapidly interconverting, low-energy conformations, including conformations that have β_{II} turns over Trp^8-Lys^9 and Thr^{10}-Phe^{11}.[10,22–27] Nuclear Overhauser enhancement (NOE) data and the temperature coefficients of selected NH chemical shifts, together with chemical shift data for C$_\alpha$H and NH protons, indicate that the mixed disulfides are interconverting between multiple conformations, some of which have secondary structure, and that some elements of the secondary structure are similar to those of the cyclic disulfide form of somatostatin.[10]

Kinetics of Intramolecular Thiol/Disulfide Exchange Reactions of Somatostatin–Glutathione Mixed Disulfides. The rate constants in Table 2 are for pH 7.00, where only a small fraction of the various thiol groups are in the reactive thiolate form. The pK_a values of 8.15 for the Cys^3 thiol group of MD1 and 9.92 for the Cys^{14} thiol group of MD2[16] indicate that a much smaller fraction of MD2 will be in the thiolate form at pH 7.0, and thus, k_{-1a} is predicted to be much less than k_{-1b}. However, rate constants for thiol/disulfide exchange also depend on the nucleophilicity of the thiolate anion,

(18) The comparison is made in terms of k_1 because values have not been reported for k_{1a} and k_{1b} for arginine vasopressin and oxytocin.

(19) The concentrations of cysteine, cysteinglglycine, and homocysteine in human plasma are in the region of 9, 3, and 0.25 μM, respectively.[9b]

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as indicated by its Bronsted basicity, and the Bronsted basicities of the central and leaving group sulfur atoms.[3]

The dependence on the Bronsted basicity of the thiolate nucleophile and the central and leaving group sulfurs is given by eq 12

$$\log k^i = C + \beta_{nuc} pK_a^{nuc} + \beta_c pK_a^c + \beta_{lg} pK_a^{lg} \quad (12)$$

where k^i is the intrinsic rate constant.[3] The constant C and the Bronsted coefficients β_{nuc}, β_c, and β_{lg} have been estimated to be 7.0, 0.50, −0.27, and −0.73, respectively, using rate data for intermolecular thiol/disulfide exchange reactions.[3] While these values for C and the Bronsted coeficients are not directly applicable to rate constants for intramolecular thiol/disulfide exchange, they can be used to account qualitatively for some features of the rate constants in Tables 2 and 3. For example, k^i_{-1a} is predicted by eq 12 to be significantly larger than k^i_{-1b}, because the Bronsted basicity of the thiolate anion of MD2 is larger and that of the central sulfur is smaller in the reaction described by k^i_{-1a}. Likewise, k^i_3 is predicted to be much larger than k^i_{-3}, as observed, because the Bronsted basicity of the thiolate nucleophile is larger and that of the leaving group is smaller in the reaction described by k^i_3. Thus, the smaller fraction of Cys^{14} in the thiolate form is compensated for by the greater nucleophilicity of the Cys^{14} thiolate anion and the dependence of thiol/disulfide exchange rate constants on the Bronsted basicity of the central and leaving group sulfur atoms, with the result that k_{-1a} and k_{-1b}, and k_3 and k_{-3}, are similar in magnitude.

Thiol/Disulfide Exchange Equilibria and the Redox Potential of the Somatostatin Disulfide Bond. Even though the disulfide bond in somatostatin is part of a much larger ring system, and the rate constants for its thiol/disulfide exchange reactions with GSH are significantly different from those of arginine vasopressin and oxytocin, the stepwise and overall equilibrium constants for its thiol/disulfide exchange reactions with GSH are essentially the same as those for arginine vasopressin and oxytocin at pH 7.00. The equilibrium constants are ratios of rate constants, i.e., $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, and $K_{ov} = (k_1k_2)/(k_{-1}k_{-2})$. Rate constant k_1 for reaction of GSH with somatostatin is significantly less than for reaction with AVP and oxytocin, but k_{-1} is also much less with the apparently fortuitous result that their ratio, and thus K_1, is essentially the same for somatostatin, AVP, and oxytocin. The rate constant for the second step in the overall reduction reaction, k_2, is the same within a factor of 2−3 for the three compounds, as is rate constant k_{-2}, as might be expected since both k_2 and k_{-2} are for thiol/disulfide exchange reactions of acyclic compounds.[6] Thus, K_2 and K_{ov} are also similar for somatostatin, AVP, and oxytocin. It also is of interest to note that the equilibrium constants are similar in magnitude to those for the reaction of two one-disulfide analogs of apamin, in each of which two of the four cysteines of apamin are replaced by serine.[28] The two cysteines in the two analogs are separated by either 9 or 11 amino acids, as compared to 10 for somatostatin.

The equilibrium constant for the overall reaction of somatostatin with GSH is related to the half-cell potential for its disulfide bond:

$$E^{\circ\prime}_{SS/SH2} = E^{\circ\prime}_{GSSG/GSH} + (RT/nF) \ln K_{ov} \quad (13)$$

A value of −0.221 V was calculated for $E^{\circ\prime}_{SS/SH2}$ using the value reported in Table 1 for K_{ov} and $E^{\circ\prime}_{GSSG/GSH}$ = −0.262 V.[29]

$E^{\circ\prime}_{SS/SH2}$ is similar to $E^{\circ\prime}$ for the disulfide bonds of AVP and oxytocin (−0.228 and −0.216 V, respectively), as expected since K_{ov} is similar for somatostatin, AVP, and oxytocin. It is also of interest to note that $E^{\circ\prime}_{SS/SH2}$ is also similar to $E^{\circ\prime}$ values reported for the disulfide bonds of octapeptide models for the active sites of the thiol−protein oxidoreductases thioredoxin, thioredoxin reductase, glutaredoxin, and protein disulfide isomerase, even though the model peptides form smaller (14-membered) disulfide-containing rings. The redox potentials of the model peptides, all of which are of the general sequence Ac-X-Cys-X-X-Cys-X-X-X-NH_2, are −0.212, −0.232, −0.237, and −0.227 V, respectively.[30] It will be of interest to determine the effect of the kinetics of the intramolecular thiol/disulfide exchange reactions on the equilibrium constants and redox potentials for the model peptides, as was done above for somatostatin, AVP, and oxytocin.

Finally, $E^{\circ\prime}$ values for a series of nonpeptide molecules that form 5−11 membered disulfide-containing rings range from −0.354 V to −0.240 V.[31] For example, the $E^{\circ\prime}$ value for dithiothreitol (DTT), lipoic acid, and 6,6′-sucrosedithiol, which form 6-, 5-, and 11-membered rings, are −0.327, −0.288, and −0.245 V, respectively. $E^{\circ\prime}$ for somatostatin is less reducing; however, it is larger than might be expected, considering that formation of the disulfide bond of somatostatin involves closure of a 38-membered ring.

Experimental Section

Chemicals. Somatostatin was obtained from Bachem, Inc., Torrance, CA. Glutathione, glutathione disulfide, dithiothreitol (DTT), and FFF were supplied by Sigma Chemical Co. HPLC-grade acetonitrile and reagent grade phosphoric acid were purchased from Fisher Scientific Co. The peptide content of the GSH, GSSG, and somatostatin was determined by 500 MHz ^{1}H NMR using an internal intensity standard. The GSH was found to be 99.7% GSH and 0.3% GSSG. The GSSG was found to be 95% GSSG; the remainder is assumed to be water because no extra resonances were observed in the ^{1}H NMR spectrum. The somatostatin was determined to be 68.7% somatostatin, with the balance being trifluoroacetate and water.

Reduced somatostatin was prepared either by reduction of somatostatin with DTT or by electrochemical reduction under nitrogen in a glove bag.[13] Somatostatin was dissolved in 0.15 M KCl to give concentrations of 20−50 μM, FFF stock solution was added, and the solution was then reduced at −1.0 V using a three-electrode system (a Hg pool working electrode, an Ag/AgCl reference electrode in saturated KCl and a Pt auxiliary electrode in 0.15 M KCl). After 25−30 min, 90−95% of the somatostatin was reduced as determined by HPLC.

Synthesis and Isolation of Mixed Disulfides. The two somatostatin−glutathione mixed disulfides were synthesized by reacting somatostatin with a mixture of GSH and GSSG. Typical concentrations were 0.5−2 mM GSSG, 1−8 mM GSH, and 100−400 μM somatostatin. Mixtures were allowed to react for ~5 min at pH 7.0 and then were quenched by lowering the pH to 2.5. The best yields of mixed disulfide were obtained with GSH:GSSG ratios of 5:1 to 10:1.

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The mixed disulfides were isolated by reversed-phase HPLC on a 10 mm × 250 mm C18 reversed-phase column. Because the mixed disulfides readily undergo intramolecular thiol/disulfide exchange reactions at neutral pH, they were isolated at pH 2.5 with a mobile phase containing 0.1% trifluoroacetic acid in acetonitrile (29%) and water. Because less than 20% of the somatostatin was converted to mixed disulfide in the reaction, the native disulfide and reduced dithiol forms of somatostatin were also collected and reacted again with a mixture of GSH and GSSG to form more mixed disulfide. The isolated mixed disulfides were lyophilized, rechromatographed to increase purity, and then stored in dry ice until used.

Kinetic and Equilibrium Studies. Reaction mixtures were analyzed by HPLC on a 3.2 mm × 100 mm C18 reversed-phase column (particle size 3 μm) and a 15 mm × 3.2 mm guard column. The detector was set at 215 nm. Chromatographic conditions were optimized by varying the pH, the percent acetonitrile, and the concentration of phosphate in the mobile phase. A pH 3.0 mobile phase containing 26% acetonitrile and 0.1 M phosphate buffer was found to give good separation of the reduced, dithiol, and mixed disulfide forms of somatostatin and the internal standard FFF. The mobile phase was prepared by adding phosphoric acid and acetonitrile to water that had been purified with a Millipore water purification system and adjusting the pH with sodium hydroxide. The mobile phase was filtered through a 0.5 μm filter to remove any particulates. The mobile phase was sparged daily with helium to remove dissolved gas to prevent postcolumn degassing, which caused a noisy base line.

The response of the detector was calibrated by measuring the ratio of analyte to internal standard peak areas vs the concentration of analyte for somatostatin and reduced somatostatin.[11] Somatostatin and reduced somatostatin concentrations ranged from 0.1 to 100 μM and 0.1 to 50 μM, respectively. The concentration of FFF was 19–21 μM. The reduced somatostatin solutions used to calibrate detector response were prepared by reducing somatostatin with DTT. Detector response was found to be linear over the above concentration ranges for somatostatin and reduced somatostatin. The limit of detection for somatostatin was determined to be ~18 nM at a signal-to-noise ratio of 3, which corresponds to ~0.36 pmol. Detector calibration factors were determined for each mixed disulfide by measuring a chromatogram for an aliquot of a solution of the mixed disulfide. The pH of the remaining solution was then increased to allow the mixed disulfide to convert to the native disulfide form by intramolecular thiol/disulfide exchange, and a chromatogram was measured for an aliquot of the solution. The calibration factor for the mixed disulfide was determined from the relative peak areas for the cyclic and mixed disulfide forms and the calibration factor for the cyclic disulfide.

All solutions used in the kinetic and equilibrium studies were deoxygenated by bubbling with oxygen-scrubbed argon through a glass dispersion tube. To exclude oxygen, experiments were conducted in a nitrogen-filled glovebag. Samples were then removed from the glovebag and analyzed by HPLC. The general procedure used in the kinetic experiments has been described previously.[7,11] To summarize the procedure for measurement of k_1, aliquots of GSH, somatostatin, and FFF standard solutions in pH 5.6–5.7 buffer (0.0375 M phosphate, 0.0375 M acetate) were combined. Samples were then removed as a function of time, quenched by lowering the pH to 2.5, and analyzed by HPLC. The procedure for determining k_{-2} was similar and involved mixing aliquots of standard solutions of GSSG, reduced somatostatin, and FFF in 0.15 M pH 7.0 buffer.

Equilibrium constants for the thiol/disulfide exchange reactions were determined at pH 7.0 in 0.075 M phosphate buffer by measuring the concentrations of the native disulfide, reduced dithiol, and mixed disulfide forms of somatostatin in solutions containing known, excess concentrations of GSH and GSSG, i.e., in solutions containing a GSH/GSSG redox buffer.[11] The procedure involved combining appropriate volumes of standard solutions; the reaction mixture was then allowed to react for 1 h, after which time an aliquot was removed, quenched, by lowering the pH with HCl to minimize air oxidation after removal from the glove bag, and analyzed by HPLC. After an additional 20 min, another aliquot was removed, quenched, and analyzed by HPLC. This procedure was repeated two more times to ensure that equilibrium was achieved, as indicated by no further change in concentration with time. The equilibrium concentrations of somatostatin, reduced somatostatin, and the two somatostatin–glutathione mixed disulfides were determined from the HPLC analysis, while the equilibrium concentrations of GSH and GSSG were calculated from their initial concentrations and the equilibrium concentrations of the various forms of somatostatin.

Kinetics experiments were conducted by combining stock solutions of the reactants, which were equilibrated at 25 °C before combining and were maintained at 25 °C throughout the experiment. Aliquots were removed as a function of time and quenched by lowering the pH to 2.5. The quenched solutions were then analyed by HPLC.

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